Cytotoxic Activity of Flavonoids and Extracts from *Retama sphaerocarpa* Boissier

Miguel López-Lázaro[a], Carmen Martín-Cordero[a], Felipe Cortés[b], Joaquín Piñero[b] and María Jesús Ayuso[a,*]

[a] Departamento de Farmacología. Facultad de Farmacia. Universidad de Sevilla, España
[b] Departamento de Biología Celular. Facultad de Biología. Universidad de Sevilla, España. Fax: 0034/95/4233765. E-mail: ayuso@fafar.us.es

* Author for correspondence and reprint requests

Z. Naturforsch. **55c,** 40–43 (2000); received July 28/September 27, 1999

Retama sphaerocarpa, Fabaceae, Flavonoids, Human Cancer Cell Lines, Cytotoxicity

Seven flavonoids isolated from chloroform, ethyl acetate and butanol extracts, obtained from the aerial parts of *Retama sphaerocarpa*, have been assessed for cytotoxic activity against three human cancer cell lines: TK-10 (renal adenocarcinoma), MCF-7 (breast adenocarcinoma) and UACC-62 (melanoma), using the SRB assay. All of them, extracts and flavonoids, were actives in, at least, one of the three cell lines at the recommended National Cancer Institute doses. They produce a dose-dependent inhibition of cell growth at concentrations in the 10^{-6}-10^{-4} M and 25–250 μg/ml range for the flavonoids and extracts respectively, being the flavonol rhamnazin the most cytotoxic.

Introduction

In the search for plants having antitumor activity, we have isolated seven flavonoids from the methanolic extract of aerial parts of *Retama sphaerocarpa*. In this present study we report the cytotoxic activity of Cl_3CH, AcOEt and BuOH extracts and seven flavonoids isolated from them: 6′-methoxypseudobaptigenin-7-β-*O*-glucoside (**1**), genistin (**2**), daidzin (**3**), orientin (**4**), rhamnazin-3-*O*-β-D-glucopyranosyl-(1→5)-[β-D-apiofuranosyl-(1→2)]-α-L-arabinofuranoside (**5**) rhamnazin-3-*O*-β-D-glucopyranosyl-(1→5)-α-L-arabinofuranoside (**6**) and rhamnazin (**7**), against three human cell lines. In addiction, the structure-activity relationship of these flavonoids is also discussed.

Materials and Methods

Plant material

The aerial parts of *R. sphaerocarpa* were collected at Zahara de la Sierra (Cádiz, Spain) in May 1996, during the flowering period. The identity was kindly verified by Dr A. Aparicio (Laboratory of Botany of the Faculty of Pharmacy, University of Sevilla) and a voucher specimen was deposited in the herbarium of this Faculty (SEV-F).

Drug tested

Flavonoids: 6′-methoxypseudobaptigenin-7-β-*O*-glucoside (**1**), genistin (**2**), daidzin (**3**), orientin (**4**), rhamnazin-3-*O*-β-D-glucopyranosyl-(1→5)-[β-D-apiofuranosyl-(1→2)]-α-L-arabinofuranoside (**5**) rhamnazin-3-*O*-β-D-glucopyranosyl-(1→5)-α-L-arabinofuranoside (**6**) and rhamnazin (**7**) were isolated of a methanolic extract from *Retama sphaerocarpa* (López-Lázaro *et al.*, 1998, 1999; Martín-Cordero *et al.*, 1999a, 1999b).

The selected method was the Netien-Lebreton (1964) technique, slightly modified by López-Lázaro *et al.* (1998): air-dried, powdered aerial parts (500 g) of *R. sphaerocarpa* were extracted by soxhlet successively for 24 h with Et_2O and for 48 h with MeOH. The MeOH extract was evaporated to dryness and suspended in 5O ml H_2O, then it was extracted successively with $CHCl_3$, EtOAc and *n*-BuOH to yield three fractions $CHCl_3$ (18 g), EtOAc (9 g) and *n*-BuOH (54 g). The dry residues obtained were fractionated by column chromatography on silicagel 60 (Merck) and Sephadex LH-20 (Pharmacia), using different proportions of ethyl acetate/methanol/water and dichloromethane/methanol as solvent systems.

Assay for cytotoxic activity

Human cell lines

The following three human cancer cell lines were used in these experiments: the human renal adenocarcinoma (TK-10), the human breast ade-

0939–5075/2000/0100–0040 $ 06.00

nocarcinoma (MCF-7) and the human melanoma (UACC-62) cell lines. They were kindly provided by Dr. G. Cragg, Department of NCI, Maryland, USA. The human tumour cytotoxicities were determined following protocols established by the National Cancer Institute, National Institute of Health (Monks *et al.*, 1985). TK-10, MCF-7 and UACC-62 cell lines were cultured in RPMI 1640 medium (Bio whittaker) containing 20% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

All cell lines were maintained at 37 °C in a 5% CO_2 atmosphere with 95% humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week. According to their growth profiles, the optimal plating densities of each cell lines was determined (15×10^3, 5×10^3 and 100×10^3 cell/well for TK-10, MCF-7 and UACC-62, respectively) to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance al 492 nm and cell number when analysed by the SRB assay.

Testing procedure and data processing

The sulphorhodamine B (SRB) assay was used in this study to assess growth inhibition. This colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye SRB (Monks *et al.*, 1985). For the assay, cells were detached with 0.1% trypsin-EDTA (Sigma) to make single-cell suspensions, and viable cells were counted using a Coulter counter and diluted with medium to give final concentracios of 15×10^4, 5×10^4 and 100×10^4 cells/ml for TK-10, MCF-7 and UACC-62 respectively. 100 μl/well of these cell suspensions were seeded in 96-well microtiter plates and incubated to allow for cell attachment. After 24 h the cells were treated with the serial concentrations of flavonoids and extracts. They were initially dissolved in an amount of 100% DMSO (40 mM) and further diluted in medium to produce 5 concentration. 100 μL/well of each concentration was added to the plates to obtain final concentration of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M for the flavonoids and for the positive controls genistein and etoposide, and 250, 25, 2.5, 0.25 and 0.025 μg/ml for the extracts. The DMSO concentration for the tested dilutions was not greater than 0.25% (vol/vol), the same as in solvent control wells. The final volume in each well was 200 μl. The plates were incubated for 48 h.

Sulphorhodamine B method

After incubating 48 h, adherent cell cultures were fixed in situ by adding 50μl of cold 50% (wt/vol) trichloroacetic acid (TCA) and incubating for 60 minutes at 4 °C. The supernatant is then discarded, and the plates are washed five times with deionized water and dried. One hundred μl of SRB solution (0.4% wt/vol in 1% acetic acid) is added to each microtiter well and the culture was incubated for 30 minutes at room temperature. Unbound SRB was removed by washing five times with 1% acetic acid. Then the plates were air-dried. Bound stain is solubilized with Tris [tris(hydroxymethyl)aminomethane] buffer, and the optical densities were read on an automated spectrophotometric plate reader at a single wavelength of 492 nm.

At the end, IC_{50} values (concentrations required to inhibit cell growth by 50%), TGI (concentration resulting in total growth inhibition) and LC_{50} (concentration causing 50% of net cell killing) were calculated according with the previously described protocols (Monk *et al.*, 1991). At least two independent experiments were carried out for each flavonoid.

Table I. Percentage of the seven flavonoids isolated from each extract.

	Cl_3CH	AcOEt	BuOH
6′-methoxypseudobaptigenin-7-β-*O*-glucoside **(1)**	1%	4.5%	–
Genistin **(2)**	0.2%	5%	–
Daidzin **(3)**	0.2%	–	–
Orientin **(4)**	–	0.2%	-
Rhamnazin-triglycoside **(5)**	–	–	0.3%
Rhamnazin-diglycoside **(6)**	–	–	1.3%
Rhamnazin **(7)**	–	–	0.3%

Results and Discussion

The results depicted in Tables II and III summarize the cytotoxic activity of the three extracts and the seven flavonoids on TK-10, MCF-7 and UACC-62 cell lines. The flavonoid genistein and the antineoplasic agent, etoposide, were taken as positive controls for comparison with the tested flavonoids and extracts. The three extracts showed

cytotoxic activity on the three cell lines at the recommended NCI (USA) doses, except BuOH extract on TK-10 cell line. However, this extract was the most cytotoxic on MCF-7 cell line. The growth of UACC-62 cells was totally inhibited by AcOEt and Cl_3CH extracts (TGI = 83 and 96 μg/ml respectively); BuOH and AcOEt extracts demonstrated total growth inhibition on MCF-7 cell line (TGI = 106 and 168 μg/ml respectively) and, on the other hand, none of the tested extracts showed total growth inhibition on TK-10 cells. Besides, AcOEt and Cl_3CH extracts on UACCC-62 cell line and BuOH extract on MCF-7 cell line produced a 50% of net cell killing (LC_{50}) at the doses of 195, 209 and 225 μg/ml respectively.

All the flavonoids isolated from these three extracts were found to possess cytotoxic activity in at least one of the three cell lines, being the no glycosylated flavonol, rhamnazin **(7)**, the most active on MCF-7 and UACC-62 cell lines, with IC_{50} values of 9.7 and 17 μM respectively. However, the most active flavonoid in the TK-10 cell line was the isoflavone genistin **(2)** (IC_{50} = 27 μM). These two flavonoids, **(2)** and **(7)**, were the only cytotoxic tested compounds against TK-10 cells, apart from the two used positive controls. On the other hand, there are, at least, one flavonoid devoid of cytotoxic activity (IC_{50}>100 μM) on TK-10, MCF-7 or UACC-62 cell lines. Therefore, they could be regarded as genuine negative indicators, testifying the specificity of the designed bioassay systems.

Bearing in mind the structures and IC_{50} values of the isoflavones genistin **(2)** and daidzin **(3)**, we can see that **(2)** is more active than **(3)** on the three studied cells lines, and the only difference is an hydroxyl group at C-5. This suggests that the C-5 hydroxyl group in isoflavones is very important for cytotoxic activity on the three tested cell lines. Relating to structure-activity relationship of these flavonoids heterosides are less cytotoxic on the three cell lines than the corresponding genines. Thus, the positive control genistein possesses more cytotoxic activity than genistin **(2)**; and the activities of the glycosylated flavonols **(5)** and **(6)** were very weak compared to the corresponding aglycon rhamnazin **(7)**. This suggests that the hydrophylic nature of sugars, or the greater volume of hetero-

Table II. Extract concentration (**μg/ml**) required to inhibit cell growth by 50% (IC_{50}), to produce total growth inhibition (TGI) and to cause 50% of net cell killing (LC_{50}).

Extracts	Inhibition parameters	TK-10	MCF-7	UACC-62
Cl_3CH	IC_{50}	87	76	42
	TGI	>250	>250	96
	LC_{50}	>250	>250	209
AcOEt	IC_{50}	49	52	36
	TGI	>250	168	83
	LC_{50}	>250	>250	195
BuOH	IC_{50}	>250	51	65
	TGI	>250	106	155
	LC_{50}	>250	225	>250

Table III. IC_{50} values (μM) of flavonoids 1–7 and positive controls tested against the cell lines TK-10, MCF-7 and UACC-62. n, number of expts.

Tested compounds	n	IC_{50} (μM) TK-10	MCF-7	UACC-62
Isoflavones				
6′-methoxypseudobaptigenin-7-β-*O*-glucóside (1)	2	>100	62	>100
Genistin (**2**)	2	27	69	27
Daidzin (**3**)	2	>100	>100	57
Flavone				
Orientin (**4**)	2	>100	>100	57
Flavonols				
Rhamnazin-triglycoside (**5**)	2	>100	62	50
Rhamnazin-diglycoside (**6**)	2	>100	49	73
Rhamnazin (**7**)[a]	2	52	9.7	17
Positive controls				
Etoposide	2	6.1	0.42	1
Genistein	2	5.9	6.9	4.1

n = number of independent experiments; [a] = López-Lázaro *et al.* 1999).

sides, could interfere with drug entering through cellular membrane. Looking for different substituent groups on the flavonoid skeleton, we can observed four methoxylated flavonoids, **(1), (5)**, **(6)**, **(7)**, and the positive control etoposide. All of them were found to possess cytotoxic activity on MCF-7 cell line, and except **(5),** this activity was more pronounced on this cell line than in the two other ones. These results indicate an appreciable degree of specificity for cytotoxic activity of methoxyl group against human adenocarcinoma cell line.

Observing Tables I, II and III we can see that the cytotoxic activity of the three tested extracts agree with the flavonoids isolated in each extract. In this way, BuOH extract was the most cytotoxic one against MCF-7 cell line, because of the presence of the methoxilated flavonols **(5)**, **(6)** and **(7)**. On TK-10 and UACC-62 cell lines, AcOEt extract was the most cytotoxic, slightly higher than Cl_3CH extract, due to the presence of the isoflavone genistin **(2)** in both extracts, being its concentration higher in the most cytotoxic one. However, we could not observed total growth inhibition (TGI) and 50% of net cell killing (LC_{50}) values in any studied flavonoid, at the NCI recommended doses, in spite of the fact that some of them possesses pronounced IC_{50} values (IC_{50} = 9.7 μM for rhamnazin on MCF-7 cell line).

Acknowledgements

The excellent technical assistance of M. A. Ledesma is very much appreciated.

We are most grateful to National Cancer Institute (NCI), USA, for the generous gift of the human cancer cell lines used in the present investigation.

We thank Programme Iberoamericano CYTED for conducting Seminaries-Taller "Bioassays in vitro".

Fresney R. I. (1987), Culture of Animal Cells. A Manual of Basic Technique. 2nd ed Alan R. Liss, New York.

López-Lázaro M., Martín-Cordero C., Iglesias-Guerra F. and Ayuso González M. J. (1998), An isoflavone glucoside from *Retama sphaerocarpa* Boissier. Phytochemistry **48**, 401–402.

López-Lázaro M., Martín-Cordero C. and Ayuso González M. J. (1999), Flavonoids of *Retama sphaerocarpa* Boissier. Plant. Med. (in press).

Martín-Cordero C., López-Lázaro M., Gil-Serrano A., Rodriguez Carvajal M. A. and Ayuso González M. J. (1999), Novel flavonol glycoside from *Retama sphaerocarpa* Boissier. Phytochemistry **51** (8), 1129–1131.

Martín-Cordero C., López-Lázaro Espartero J. L. and Ayuso González M. J. (1999), Retamatrioside, a new flavonol triglycoside from *Retama sphaerocarpa* Boissier. J. Nat. Prod. (in press).

Monks A., Scudeiro D., Skehan P., Shoemaker R., Paull K., Vistica D., Hose C., Langley J., Cronise P., Vaigro-Wolff A., Gray-Goodrich M., Campbell H., Mayo J. and Boyd M. (1991), Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J. Natl. Cancer Inst. **83**, 757–766.

Netien G. and Lebreton P. H. (1964), Sur les flavonoides et autres substances polyphenoliques du *Millepertius hypericum* nummularium L. Ann. Pharm. Fr. **22**, 69–79.