Cytotoxic Triterpenoids from *Erica andevalensis*

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Human Cancer Cell Lines, Triterpenoids, Ursolic Acid

The cytotoxic activity of two pentacyclic triterpenoids (ursolic acid and α-amyrine) isolated from the methanolic extract of the aerial parts from *Erica andevalensis*, whose structures have been established on the basis of spectroscopic and chemical evidence, has been assessed against three human cancer cell lines, TK-10 (renal adenocarcinoma), MCF-7 (breast adenocarcinoma) and UACC-62 (melanoma), recommended by NCI (National Cancer Institute) and we also evaluated the antimitotic effect in root meristematic cells of *Allium cepa*. Ursolic acid was found to possess the highest cytotoxic activity.

Introduction

Triterpenoids exist widely in nature and are the major components of some traditional medicinal plants. The biological significance of triterpenoid compounds has been review (Mahato *et al*., 1988; Price *et al*., 1987), and the interest in these compounds is growing. Triterpenoids have a variety of biological effects such as antiinflammatory, antihyperlipemia, anti-ulcer, hepatoprotective, skin-tumor prevention and immunomodulatoty effects (Hu, 1988; Nishino *et al*., 1988; Mahato *et al*., 1988; Price *et al*., 1987). Ursolic acid, a steroid-like triterpene compound, is reported to produce a wide variety of pharmacological activities (Es-saady *et al*., 1994; Lee *et al*., 1994; Umehara *et al*., 1992; Liu *et al*., 1995; Ringbom *et al*., 1998).

Erica andevalensis Cabezudo-Rivera (Ericaceae) is a perennial plant widespread in the Andévalo (Huelva) which has been used in folk medicine as an urinary antiseptic. In the previous study on the antibacterial, antiulcer, antimitotic, and diuretic constituents, we isolated phenolics acids and flavonoids (Aumente *et al*., 1988; Toro *et al.,* 1987, 1988; Pascual *et al*. 1987; Reyes *et al*., 1996a, 1996b).

As part of our continuing study of *Erica andevalensis* for novel compounds which might possess biological activity, we have evaluated the antimitotic effect of the methanolic extract and the bioactive constituents, and isolated and identified two triterpenoids: ursolic acid (**1**) and α-amyrine (**2**) and examined their cytotoxic activity against three tumor cell lines, TK-10 (renal adenocarcinoma), MCF-7 (breast adenocarcinoma) and UACC-62 (melanoma), recommended by NCI, using the SRB (sulphorhodamine B) assay. Their structures have been established on the basis of spectroscopic and chemical evidence.

Material and Methods

Plant material

The aerial parts of *Erica andevalensis* were collected in Nerva (Huelva, Spain), and identified by Dr A. Aparicio (Laboratory of Botany of the Faculty of Pharmacy, University of Sevilla). A voucher specimen is deposited, labelled SEVF.

Isolation of the cytotoxic constituents

Aerial parts (500 g) were extracted by maceration with MeOH (2.5 l), and the extract was evaporated to dryness (30 g). The MeOH extract (4 g) was chromatographed on Silica gel with increasing concentration of EtOAc in n-hexane as the eluting solvent. Fractions were monitored by TLC (Si gel benzene-EtOAc 2:1 v/v, visualized with oleum reagent, sulfuric acid/acetic acid/water 2:40:8 v/v, and heat). The triterpenoids compounds were purified by rechromatography over a small column and recrystallization from MeOH (ursolic acid: 105 mg, α-amyrine: 32 mg). The identification of the isolated compounds was performed by physical and spectroscopic methods: mp, IR, MS, ^{1}H NMR.

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Assays for cytotoxic activity

A) The root meristematic cells *Allium cepa* L were treated according to the method described by Levan and Lotfy (1949). Briefly, germinating onion bulbs (root length of 20–30 mm) were transferred to the tubes with dimethylsulfoxide (DMSO) at 1% (Cortés *et al.*,1987) as solvent. The tested doses were 0.25, 0.5 and 1 g/100 ml for different treatment times (4, 8, 24, 48, and 72 h). The root tips were then cut off, washed in distilled water and fixed in AcOH-EtOH 1:3 v/v at 5 °C overnight (Wilson and Morrison, 1971); subsequently, roots were stained with acetic-orcein (Tjio and Levan, 1950). Controls were obtained by allowing bulbs to grown in distilled water for the same times. Experiments were done in duplicate, using 3 bulbs for each assay. For the calculation of mitotic index (% cells in division) and phases index (% cells in a mitotic phase), at least 2000 cells were scored in each case.

B) *Human tumor cell lines*: Human cell lines: The following three human cancer cell lines were used in these experiments: the human renal adenocarcinoma (TK-10), the human breast adenocarcinoma (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.* 1991).

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 line was determined (15×10^3, 5×10^3 and 100×10^3 cells/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 at 492 nm and cell number when analysed by the SRB assay. Data for isolated compounds were performed out according to the standard protocols established by the NCI (Monks *et al.*,1991).

Results and Discussion

The identification of the two triterpenoids, ursolic acid and α-amyrine isolated from the methanolic extract of the aerial parts from *Erica andevalensis*, were based on the comparison of physical and spectral data (mp, IR, MS, ^{1}H NMR) with data registered in the literature (Mulkherjee *et al.*, 1982; Piozzi *et al.*, 1986; Lee *et al.*, 1988; Simon *et al.*, 1992).

The methanolic extract was found to have an antimitotic effect. When the root meristem cells were grown in the presence of 1, 0.5 and 0.25%

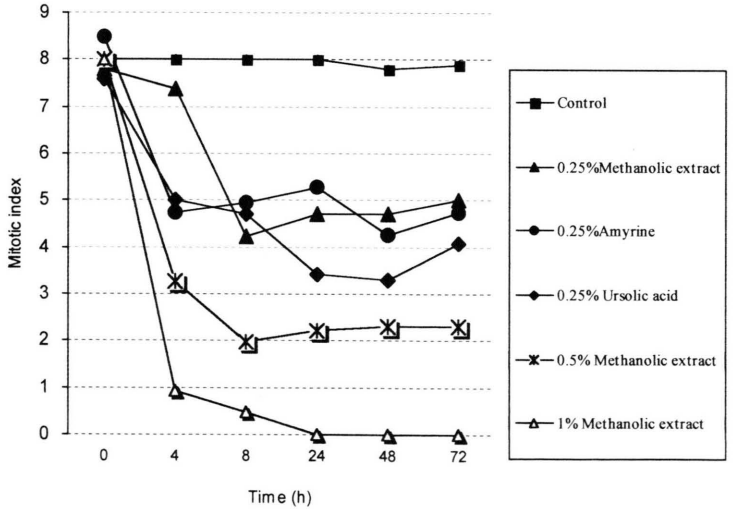


Fig. 1. Evolution of mitotic index when *Allium cepa* was treated with 0.25, 0.5 and 1 g/100 ml methanolic extract and 0.25 g/100 ml of ursolic acid and α-amyrine.

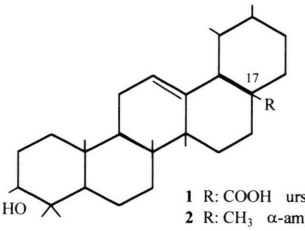
methanolic extract, the mitotic index decreased below control values (Fig. 1). The decrease in the mitotic index was dose-dependent with the maximun effect at 1 g dry wt/100 ml. At this dose the mitotic index is nearly zero after 24 h of treatment. The ursolic acid and α-amyrine produced a marked mitodepressive effect on *Allium cepa* root meristems (Fig. 1). This finding suggests that there are two bioactive components essential for the antimitotic effect of *Erica andevalensis.* The methylated triterpenoid, α-amyrine, was found to be less active than ursolic acid. The phase indices (% cells in a mitotic phase) calculated are normal; indicating that these triterpenoids exerted their antimitotic effect on the interphase. These findings agree with the cytostatic activity reported for ursolic acid (Es-saady *et al.*, 1996a, 1996b), which blocked cells in the G1 phase of the cell cycle. The *Allium* assay is a valid standard method for testing plant extracts, with low cost and good correlation to mammalian test systems.

The results obtained on cytotoxic activity against three cultured tumor cell lines are summarized by Table I. Ursolic acid was found to possess pronounced activity against the three cancer cell lines with GI_{50} values in the range of 11.2×10^{-3} and 15.4×10^{-3} μmol/ml with no discernible cell type selectivity. This indicates that in comparison with the corresponding methylated triterpenoid α-amyrine, the acid substitution at C-17 in ursolic acid is essential for cytotoxic activity. Under the same test conditions, etoposide (4′-desmethylepipodophyllotoxin 9-[4,6-O-ethylidene-β-$_D$-glucopyranoside], the positive control) (Topogen) was found to be cytotoxic against the three cancer cell lines with a GI_{50} in the range of 0.4×10^{-3} and 6.1×10^{-3} μmol/ml. Ursolic acid has previously been shown as a potential antitumor agent (Ohigashi *et al.*, 1986; Tokuda *et al.*, 1986), it induced apoptosis, stabilized liposomal membranes, recovered the hematopoietic system and is an inhibitor of human DNA ligase I.

Table I. Cytotoxic activity of ursolic acic and α-amyrine against three cultured tumor cell lines[a].

Compounds	Inhibition parameters	TK-10	MCF-7	UACC-62
Ursolic acid	GI_{50}	$15.4\ .10^{-3}$	$11.2\ .10^{-3}$	$11.8\ .10^{-3}$
	TGI	$28.5\ .10^{-3}$	$26.3\ .10^{-3}$	$30.7\ 10^{-3}$
	LC_{50}	$52.6\ .10^{-3}$	$61.4\ .10^{-3}$	$81.1\ .10^{-3}$
α-Amyrine	GI_{50}	$51.6\ .10^{-3}$	$22.5\ .10^{-3}$	$23.5\ .10^{-3}$
	TGI	$15.3\ 10^{-3}$	$63.4\ .10^{-3}$	$86.8\ .10^{-3}$
	LC_{50}	$453.0\ .10^{-3}$	$171.3.10^{-3}$	$305.1\ .10^{-3}$
Etoposide	GI_{50}	$6.1.10^{-3}$	$0.4.10^{-3}$	$1.0\ .10^{-3}$
	TGI	$27.8\ .10^{-3}$	100.10^{-3}	$30.5\ .10^{-3}$
	LC_{50}	$97.3\ .10^{-3}$	$100\ .10^{-3}$	$100\ .10^{-3}$

[a] Concentration (μmol/ml) required to inhibit cell growth by 50% (GI_{50}), to produce total growth inhibition (TGI) and to cause 50% of net cell killing (LC_{50}).



1 R: COOH ursolic acid
2 R: CH_3 α-amyrine

Fig. 2. Structures of α-amyrine and ursolic acid.

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