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RESEARCH PAPER

Cytotoxic activity of polyacetylenes and polyenes isolated from roots of *Echinacea pallida*

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Background and purpose: The *n*-hexane extracts of the roots of three medicinally used *Echinacea* species exhibited cytotoxic activity on human cancer cell lines, with *Echinacea pallida* found to be the most cytotoxic. Acetylenes are present in *E. pallida* lipophilic extracts but essentially absent in extracts from the other two species. In the present study, the cytotoxic effects of five compounds, two polyacetylenes (namely, 8-hydroxy-pentadeca-(9*E*)-ene-11,13-diyn-2-one (**1**) and pentadeca-(9*E*)-ene-11,13-diyne-2,8-dione (**3**)) and three polyenes (namely, 8-hydroxy-pentadeca-(9*E*,13*Z*)-dien-11-yn-2-one (**2**), pentadeca-(9*E*,13*Z*)-dien-11-yne-2,8-dione (**4**) and pentadeca-(8*Z*,13*Z*)-dien-11-yn-2-one (**5**)), isolated from the *n*-hexane extract of *E. pallida* roots by bioassay-guided fractionation, were investigated and the potential bioavailability of these compounds in the extract was studied.
Experimental approach: Cytotoxic effects were assessed on human pancreatic MIA PaCa-2 and colonic COLO320 cancer cell lines. Cell viability was evaluated by the WST-1 assay and apoptotic cell death by the cytosolic internucleosomal DNA enrichment and the caspase 3/7 activity tests. Caco-2 cell monolayers were used to assess the potential bioavailability of the acetylenes.
Key results: The five compounds exhibited concentration-dependent cytotoxicity in both cell types, with a greater potency in the colonic cancer cells. Apoptotic cell death was found to be involved in the cytotoxic effect of the most active, compound **5**. Compounds **2** and **5** were found to cross the Caco-2 monolayer with apparent permeabilities above 10×10^{-6} cm s^{-1}.
Conclusions and implications: Compounds isolated from *n*-hexane extracts of *E. pallida* roots have a direct cytotoxicity on cancer cells and good potential for absorption in humans when taken orally.
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Keywords: *Echinacea*; acetylenes; cancer; MIA PaCa-2; COLO320; Caco-2; permeability; cytotoxicity

Abbreviations: ClogP, calculated log octanol/water partition coefficient; Caco-2, human colon carcinoma epithelial cell line; COLO320, human colorectal adenocarcinoma cell line; HEK-293, human embryonic kidney cell line; MIA PaCa-2, human pancreatic adenocarcinoma cell line; P_{app}, apparent permeability; SASA, solvent-accessible surface area; TEER, transepithelial electrical resistance; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulphonate

Introduction

Echinacea is one of the most popular medicinal herbs and today *Echinacea* products are among the best-selling herbal preparations in the industrialized world. The genus *Echinacea* (Asteraceae family) consists of nine species of which only three are traditionally used in medicine: *Echinacea purpurea* (L.) Moench, *E. angustifolia* (DC.) Hell. and *E. pallida* (Nutt.) Nutt.

Echinacea has a long history of medicinal use for the treatment of the common cold, upper respiratory infections and a number of other disease conditions (Barnes *et al.*, 2005). An extremely complex chemical composition has been observed in the *Echinacea* genus, including caffeic acid derivatives (Cheminat *et al.*, 1988; Pellati *et al.*, 2004, 2005), alkamides, acetylenes (polyacetylenes and polyenes) (Bauer *et al.*, 1988a; Bauer and Remiger, 1989; Pellati *et al.*, 2007), polysaccharides (Wagner *et al.*, 1988) and glycoproteins (Classen *et al.*, 2000; Thude and Classen, 2005), all of which

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exhibit diverse pharmacological activities. Recent attention has focused particularly on alkamides that are lipophilic constituents with cannabinomimetic activity (Woelkart *et al*., 2005; Raduner *et al*., 2006), interaction with the immune system (Gertsch *et al*., 2004) and good bioavailability following oral administration in human (Matthias *et al*., 2005). In contrast, little information is available about the activity of the acetylenic compounds from *Echinacea*.

We have recently reported the potential for anticancer activity by *Echinacea* extracts related to the *in vitro* cytotoxic and pro-apoptotic properties of *n*-hexane root extracts from the three medicinally important *Echinacea* species (Chicca *et al*., 2007). A more pronounced cytotoxic effect for *E. pallida* root extracts was observed. This correlates with the different chemical profile of the lipophilic constituents in each of the species, with *E. purpurea* and *E. angustifolia* having alkamides as the main lipophilic constituents (Bauer *et al*., 1988a, b, 1989; Bauer and Remiger, 1989), whereas *E. pallida* contains polyacetylenes and polyenes (Pellati *et al*., 2007).

More than 1000 acetylenic molecules have been isolated from plants, fungi, microorganisms and marine invertebrates and identified. The biological activities of acetylenes and related compounds have been studied intensively in recent years and their activity in various organisms is now well documented (Dembitsky, 2006). Polyacetylenes of different sources have been proven to be cytotoxic against different types of cancer cell lines (Matsunaga *et al*., 1990; Lim *et al*., 1999; Ito *et al*., 2001; Lee *et al*., 2004; Whelan and Ryan, 2004; Zidorn *et al*., 2005; Choi *et al*., 2006; Park *et al*., 2006) and to enhance the cytotoxic activity of other anticancer drugs (Matsunaga *et al*., 1994).

The aim of the present study was to evaluate the cytotoxic activity of polyacetylenes and polyenes from *E. pallida* roots, isolated through bioassay-guided fractionation, on two human cancer cell lines, the adenocarcinoma MIA PaCa-2 (human pancreatic adenocarcinoma cell line) and the colonic cell line, COLO320 (human colorectal adenocarcinoma cell line). The potential bioavailability of the *E. pallida* lipophilic compounds was also investigated using Caco-2 (human colon carcinoma epithelial cell line) cell monolayers.

Materials and methods

Drugs and plant material

5-Fluorouracil was purchased from Sigma-Aldrich (Milan, Italy). Authentic dried roots (1 kg) of 3-year-old *E. pallida* (Nutt.) Nutt. were purchased from Planta Medica s.r.l., Pistrino, Perugia, Italy, in January 2005. A voucher specimen was deposited at the Herbarium of the Botanical Garden of the University of Modena and Reggio Emilia (Italy). The plant material was kept in the dark, protected from high temperature and humidity, until required for extraction. The roots were ground with an IKA M20 grinder immediately before extraction.

An alkamide containing *Echinacea* extract (Echinacea Premium Liquid) was obtained from MediHerb Pty Ltd (Warwick, Australia) and used as a reference in the bioavailability studies as it had been previously shown to possess both bioavailable and non-bioavailable compounds (Matthias *et al*., 2004).

Extraction and isolation of compounds

Powdered dried roots of *E. pallida* (1 kg) were extracted in a Soxhlet apparatus for 24 h using *n*-hexane (5.4 l). The extract was evaporated to dryness under vacuum to give a yellow oil (8 g). The extract was subjected to silica gel column chromatography, affording 155 fractions. Each fraction was analysed by TLC and reversed phase-HPLC, and combined into 10 fractions according to their chromatographic profile. From these fractions, the purified compounds (**1–5**) (Figure 1) were isolated by silica gel column chromatography and preparative TLC. The structures of the compounds were determined on the basis of UV, IR, NMR (including 1D and 2D NMR experiments, such as ^{1}H–^{1}H gCOSY, gHSQC-DEPT, gHMBC, gNOESY) and MS spectroscopic data (Pellati *et al*., 2006). Purified compounds were stored under argon at low temperature (−20 °C), protected from light and humidity.

Sample preparation for bioavailability study

Powdered dried roots of *E. pallida* (40 g) were extracted in a Soxhlet apparatus for 4 h using *n*-hexane (200 ml). The extract was evaporated to dryness under vacuum to give a yellow oil (400 mg). The crude extract was stored under argon at low temperature (−20 °C), protected from light and humidity.

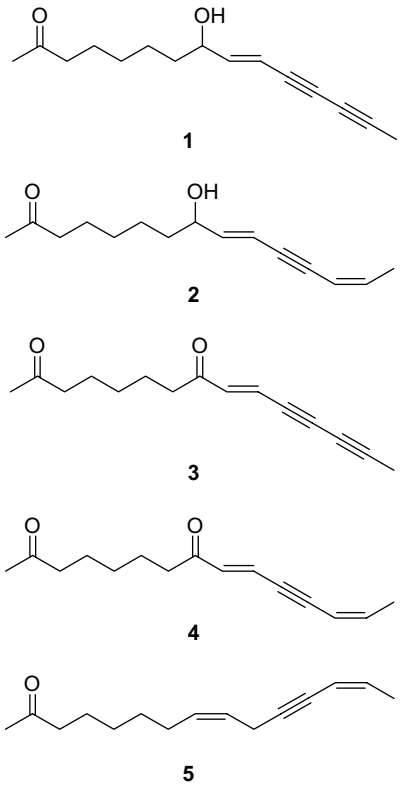

Figure 1 Chemical structures of the compounds isolated from *E. pallida* roots.

Cell lines and culture

MIA PaCa-2, COLO320, Caco-2 and the human embryonic kidney HEK-293 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). MIA PaCa-2 and HEK-293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM), 10% fetal bovine serum, 2.5% horse serum, 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (Sigma-Aldrich). COLO320 were cultured in RPMI 1640 medium supplemented with L-glutamine (2 mM), 10% fetal bovine serum, 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (Sigma-Aldrich). Caco-2 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% non-essential amino acids as previously described (Matthias *et al*., 2004). All four cell lines were maintained at 37 °C in humidified incubator under 5% CO_2. Cell plates and transwell polycarbonate inserts were from Costar (Cambridge, MA, USA). Cell culture reagents were purchased from Gibco-BRL (Gran Island, NY, USA).

Cell viability assay

Cell viability was assessed using the Cell Proliferation Reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulphonate) (Roche, Mannheim, Germany) based on the cellular cleavage of the WST-1 to formazan. Cells were seeded onto 96-well plates at a density of 2×10^3 per well and incubated at 37 °C with 5% CO_2. After 24 h incubation to allow cell attachment, cells were treated for 72 h with the acetylenic compounds in the range 0.1–100 μM. At the end of the exposure time, WST-1 was added at 1/10 of the total volume and after 60 min of incubation at 37 °C, the absorbance was measured at 450 nm with a microplate reader (Wallac; PerkinElmer, Wellesley, MA, USA).

Inhibition of cell viability was assessed as the percentage reduction of ultraviolet absorbance of treated cells versus control cultures (vehicle-treated cells) and the 50% inhibitory concentration of cell growth (IC_{50}) was calculated by nonlinear least squares curve fitting (GraphPad Software, San Diego, CA, USA). Each value was obtained from three independent experiments carried out in triplicate.

Caspase 3/7 activity assay

Caspase 3/7 activities were assayed by the Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega, Milan, Italy), according to the manufacturer's instructions. Briefly, cells were plated onto 96-well plates (6×10^3 per well) in the presence of *E. pallida* compounds **1–5** for 24 h. After cell lysis, the caspase 3/7 assay substrate ((Z-DEVD)2-Rhodamine 110) was added and the fluorescence measured (Wallac; PerkinElmer) at excitation and emission wavelengths of 485 and 530 nm, respectively. Values were expressed as the ratio between fluorescent signals generated in cells treated with the compounds and those produced in vehicle-treated cells. Each value was obtained from three independent experiments carried out in triplicate.

DNA fragmentation assay

The occurrence of the nuclear DNA fragmentation was assessed by the Cell Death Detection ELISA^{PLUS} kit (Roche), which qualitatively and quantitatively detects the amount of cleaved DNA/histone complexes (nucleosomes) by using a sandwich-enzyme-immunoassay-based method. Briefly, after treatment with the *E. pallida* compounds **1–5** for 24 h, cells were pelleted and the supernatant was carefully removed to analyse necrosis while the cell pellet was lysed to produce nucleosomes. Subsequently, an aliquot of the lysate was transferred to streptavidin-coated microplates, incubated with immunoreagent (containing anti-histone and anti-DNA antibodies) and peroxidase substrate ABTS (2,2-azino-di (3-ethylbenzthiazolin-sulphonate)). The resulting colour development, which was proportional to the amount of nucleosomes captured in the antibody sandwich, was measured spectrophotometrically at 405 nm (Wallac; PerkinElmer). Data were normalized against the control (vehicle-treated cells) and results are presented as the mean from three independent experiments carried out in triplicate.

Diffusion through Caco-2 cell monolayers

Transepithelial electrical resistance (TEER) of the monolayers was measured using the Millicell-ERS system (Millipore Corp., Bedford, MA, USA) before and after transport experiments to determine the integrity of the monolayers.

At the start of the experiments, 100 μl of Hanks balanced salt solution-HEPES containing the test preparation was added to the apical and 600 μl of Hanks balanced salt solution-HEPES was added to the basolateral side of the monolayers. The plates were shaken in a Heifolf Titramax 1000 at 400 r.p.m. at 37 °C throughout the experiment. At 10, 20, 30, 60, 90, 120 and 150 min, the basolateral volume was removed and replaced with fresh Hanks balanced salt solution-HEPES. The apical solution was sampled only at the conclusion of the experiment. Stock solutions were made in DMSO and then diluted in HEPES buffer to give a final concentration of 0.2% DMSO added to the apical side of the monolayers.

Acetylene concentrations in the samples were determined using a Shimadzu gradient HPLC system (Shimadzu LC10AT) coupled to a quadrupole mass spectrometer (Shimadzu 2010-EV) operating in positive ion mode using an atmospheric pressure chemical ionization interface. The mobile phase was a mixture of water (A) and acetonitrile (B), both containing 0.1% formic acid. The elution gradient used was 40–100% B over 25 min, followed by a period of re-equilibration at 40% B prior to the next injection. A Phenomonex C_{18}, 3 μm, 100 mm × 2.00 mm column was used with a solvent flow rate of 0.3 ml min^{-1}. Apparent permeability coefficients (P_{app}, cm s^{-1}) were determined at 90 min as previously described (Matthias *et al*., 2004). Three replicates of each test solution were performed.

Computational analysis

The molecular structures of the compounds isolated from *E. pallida* were built and graphically handled by means of the InsightII package (MSI-Accelrys) (Biosym/MSI, San Diego, CA, USA, 1995). Molecular geometries were optimized

through molecular mechanics-based energy minimizations, by using the Discover program (MSI-Accelrys) (Biosym/MSI), where the cff91 force field was selected. To reasonably simulate plausible conformations of the *E. pallida* compounds in aqueous environment, all the molecular models were solvated by surrounding them with 18-Å radius spheres of water molecules. The hydrated molecules then underwent energy minimization according to the protocol described below: at first 100 interactions, performed by using a steepest descent algorithm, allowed the system energy to rapidly decrease; then a conjugate gradient algorithm was exploited until a pre-imposed convergence criterion was satisfied (maximum derivative less than 0.001 kcal $Å^{-1}$).

On the conformers obtained for each molecule after optimizations, solvent-accessible surface area (SASA) calculations were performed using the Homology module of InsightII (Biosym/MSI), whereas molecular weights, volumes and ClogP (calculated log octanol/water partition coefficient) were computed (data not shown) with Chem3D Ultra of the ChemOffice program (Cambridge-Soft Corporation). The two enantiomers (*R* and *S*) for each racemic mixture (compounds **1** and **2**) were considered and their calculated properties were averaged.

Data analysis

Data are presented as mean values ± s.e. The statistical significance of the differences between means was evaluated by Student's *t*-test for unpaired data. $P<0.05$ was taken to be significant.

Results

Cytotoxic activity of polyacetylenes and polyenes from E. pallida roots

Compounds **1–5** caused a significant concentration-dependent decrease in MIA PaCa-2 and COLO320 cell viability after 72 h exposure, but had no cytotoxic effect against the non-tumour embryonic kidney cells HEK-293. The IC_{50} values reported in Table 1 show compound **5** to be the most cytotoxic. Moreover, COLO320 appears to be more sensitive than MIA PaCa-2 cells to all the isolated compounds. In this cell line, the IC_{50} value of compound **5** was significantly lower than that of the reference drug 5-fluorouracil.

Cell death mechanism involved in compound 5 cytotoxicity

To gain insights into the mechanism involved in the cytotoxicity induced by the most active constituent, compound **5**, caspase 3/7 activity and the level of cytosolic histone-associated DNA fragments were evaluated. After 24 h of incubation with the IC_{50} concentrations in MIA PaCa-2 and COLO320, cells respectively, the caspase 3/7 activity significantly increased (1.51 ± 0.05- and 1.47 ± 0.05-fold higher than vehicle-treated cells in MIA PaCa-2 and COLO320 cells, respectively) (Figure 2a). Moreover, the cytosolic internucleosomal DNA fragments were significantly enhanced as well (2.97 ± 0.25- and 2.32 ± 0.45-fold higher than vehicle-treated cells in MIA PaCa-2 and COLO320 cells, respectively). In contrast, no extracellular DNA fragments were detected for both cell lines in this experimental setting (Figure 2b).

Table 1 IC_{50} values of the purified compounds isolated from *E. pallida* roots on MIA PaCa-2 and COLO320 cells, calculated after 72 h exposure

Compounds	*IC_{50} (μM), mean ± s.e.*	
	MIA PaCa-2	*COLO320*
1	>100	80.13 ± 2.21
2	>100	21.77 ± 1.22
3	60.91 ± 0.61	25.28 ± 0.55
4	63.53 ± 1.12	22.84 ± 2.12
5	32.17 ± 3.98	2.34 ± 0.33
5-FU	7.42 ± 0.55	8.72 ± 0.18

Abbreviations: COLO320, human colorectal adenocarcinoma cell line; MIA PaCa-2, human pancreatic adenocarcinoma cell line.
Data are means from three independent experiments each run in triplicate.

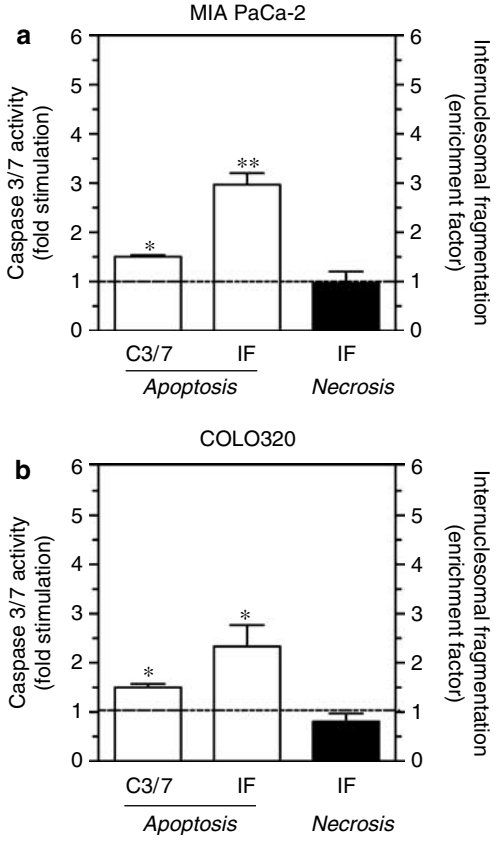

Figure 2 Apoptotic and necrotic cell death after treatment with compound **5** for 24 h on MIA PaCa-2 (30 μM) (**a**) and COLO320 (2.5 μM) (**b**) cells. Caspase 3/7 activity (C3/7) and internucleosomal fragmentation (IF) were expressed as fold stimulation and as enrichment factor of nucleosomal fragments versus untreated cells, respectively. Data are means ± s.e. from three independent experiments each run in triplicate. *$P<0.05$; **$P<0.01$, versus untreated cells.

Diffusion of polyacetylenes and polyenes through Caco-2 cell monolayers

The integrity of the Caco-2 monolayers was determined at the beginning and at the end of the experiment. Control TEER values were greater than 500 Ω cm^2, indicating that the

membranes were intact at all stages of the experiment. TEER values of the monolayers exposed to the *E. pallida* acetylenes decreased from $560 \pm 50\,\Omega\,cm^2$ at the beginning of the experiment to $460 \pm 12\,\Omega\,cm^2$ at the end. Some monolayers decreased their TEER values to approximately $200\,\Omega\,cm^2$ but there was no correlation between *E. pallida* preparation and TEER decreases. To ensure the cells were fully differentiated and confluent, cichoric acid in the Echinacea Premium preparation was used as a negative control as it has been previously shown to have low P_{app}. P_{app} for cichoric acid was found to be $0.5 \times 10^{-6}\,cm\,s^{-1}$, which is in good agreement with previously reported values (Matthias *et al*., 2004).

Of the acetylenes present in the *E. pallida* extracts (Pellati *et al*., 2007), only three acetylenes were found to be potentially bioavailable, compounds **2**, **5** and the tetradec-(8*Z*)-ene-11,13-diyn-2-one (compound **6**), which was excluded from the cytotoxicity assay as it was present in fractions not indicated as potentially active during the bioassay-guided fractionation. Transport kinetics for the three acetylenes are shown in Figure 3. The three acetylenes readily crossed the Caco-2 monolayers, although their permeability across the monolayer varied greatly. The calculated P_{app} values are given in Table 2.

Computational analysis

Due to the small number of compounds, only a quite elemental structure–activity study based on the use of suitable molecular descriptors can be undertaken. Some molecular descriptors related to the size, shape and lipophilicity of the molecules were considered to be more strictly related to the molecular features expected to account for the activity of the analysed compounds: molecular weights, molecular volumes, ClogP and SASA. While the simplest descriptors, such as molecular weights, molecular volumes, ClogP, were not able to explain the trend of the biological activity, more complex descriptors, such as the two contributions of the SASA (SASA polar and nonpolar components as well as combinations of them) showed a trend quite similar to that shown by the experimental activity data (Table 3).

In particular, when data obtained on MIA PaCa-2 cells were analysed, the polar component of SASA (for images, see Supplementary data) was observed to follow a trend in accordance with the experimental IC_{50} values. Lower values of polar SASA correspond to lower IC_{50} values (higher activity). On the other hand, in COLO320 cells, a good correlation was given by the nonpolar component of the SASA and activity: the most active compound (**5**) showed the highest values of nonpolar SASA, whereas less active compounds showed lower values of this molecular descriptor (Table 3).

Discussion and conclusions

Over 60% of currently used anticancer agents are of natural origin, derived from plants, marine organisms and microorganisms (Cragg and Newman, 2005). Cytotoxicity screening by using *in vitro* cell culture is a useful tool for the discovery of new potential anticancer agents from natural products. The criteria of cytotoxic activity for the crude extracts, as established by the American National Cancer Institute, is an $IC_{50} < 30\,\mu g\,ml^{-1}$, which legitimates further investigation on single active constituents (Suffness and Pezzuto, 1990). In a previous study (Chicca *et al*., 2007), evaluation of the cytotoxicity of the *n*-hexane extracts from the three medicinally used *Echinacea* species revealed a

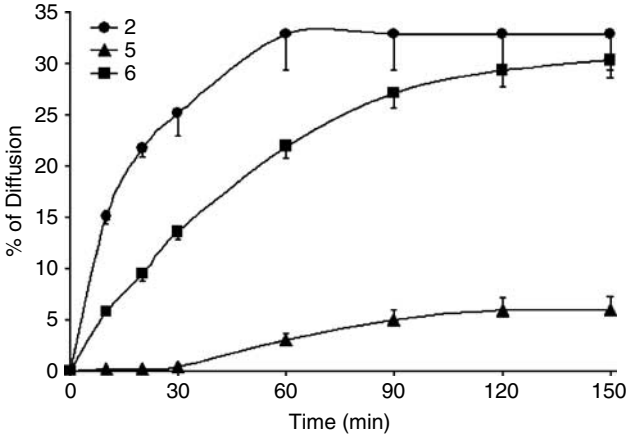

Figure 3 Transport kinetics of acetylenes in the *E. pallida* root extract through Caco-2 monolayers during a 2.5 h incubation. Values are means ± s.e. from three preparations each run in triplicate. Compound **2**: 8-hydroxy-pentadeca-(9*E*,13*Z*)-dien-11-yn-2-one; compound **5**: pentadeca-(8*Z*,13*Z*)dien-11-yn-2-one; compound **6**: tetradec-(8*Z*)-ene-11,13-diyn-2-one.

Table 2 Acetylene permeability across Caco-2 monolayers after 90 min

Compounds	*Apparent permeability* ($\times 10^{-6}\,cm\,s^{-1}$)
2	49 ± 7
5	10 ± 3
6	32 ± 3
AA mw = 247	57 ± 3
AA mw = 231	158 ± 31
Cichoric acid	0.48 ± 0.11

Abbreviation: Caco-2, human colon carcinoma epithelial cell line.
Data are means ± s.e. from three preparations each run in triplicate. (**2**, **5**, **6** = acetylenes from *E. pallida*; alkamides (AA) and cichoric acid were from *E. angustifolia* and *E. purpurea* preparation as previously reported (Matthias *et al*., 2004).

Table 3 Total SASA and its components calculated for each compound tested on MIA PaCa-2 and COLO320 cells

Compounds	*Total SASA (Å)*	*Polar SASA (Å)*	*Nonpolar SASA (Å²)*	*Nonpolar/ polar ratio*
1[a]	564.08	83.57	480.51	5.79
2[a]	577.86	78.17	499.69	6.48
3	554.13	66.31	487.82	7.36
4	561.56	66.55	495.02	7.44
5	550.82	38.30	512.53	13.38

Abbreviations: COLO320, human colorectal adenocarcinoma cell line; MIA PaCa-2, human pancreatic adenocarcinoma cell line; SASA, solvent-accessible surface area.
[a]For the two enantiomers of each racemic mixture, averaged values are reported.

suitable average IC_{50} ($28.5\,\mu g\,ml^{-1}$) concentration in two human cancer cell lines for *E. pallida* extracts. Bioassay-guided fractionation then allowed isolation of five compounds from the *E. pallida n*-hexane root extract. In agreement with a number of other studies on the activity of acetylenic compounds on different cancer cell lines (Dembitsky, 2006), the *E. pallida* constituents exhibited a concentration-dependent cytotoxicity against both the pancreatic MIA PaCa-2 and the colonic COLO320 human cancer cell lines. These compounds showed a selectivity effect on cancer cells versus non-tumour cells with an IC_{50} higher than 100 μM found in cytotoxic assays against HEK-293 cells. Compound **5** was the most active of the investigated compounds with a very low IC_{50} value particularly in the colonic cell line (approximately 2 μM). For all the tested compounds, greater sensitivity in COLO320 cells was observed, according to the results obtained with the crude lipophilic extracts in our previous work (Chicca *et al*., 2007). Nonetheless, the results obtained with compound **5** seem to be relevant also in MIA PaCa-2 cells, because of the general low sensitivity of this cell type to therapeutic agents (Yang *et al*., 2003).

Analysis of the molecular structure of compound **5** indicated only small conformational differences between it and the other compounds, but the balance between polar and nonpolar regions could be of relevance in explaining its greater activity. In fact, in its structure there is a greater prevalence of nonpolar areas. This property may influence its permeability through cell membranes and gives some advantages in interactions with the molecular target(s) of the acetylene. Unfortunately, until now, a clear mechanism of action for this compound could not be described.

Different mechanisms have been suggested for other cytotoxic acetylenic compounds described in the literature. For example, nonspecific injury to the cytoplasmic membrane, nuclear envelope and mitochondria of L1210 cells by the polyacetylenes of *Panax ginseng* has been reported, with a minor activity by the relatively polar panaxytriol (Kim *et al*., 1988). On the other hand, specific activities, such as inhibition of COX and lipoxygenase enzymes, inhibition of DNA synthesis and NO production, have been suggested (Dembitsky, 2006). Moreover, arrest of cell cycle progression in various phases and apoptosis induction are other potential mechanisms (Kuo *et al*., 2002; Choi *et al*., 2006; Dembitsky, 2006; Park *et al*., 2006).

Induction of apoptosis in cancer cells is one of the useful strategies for anticancer drug development (Hu and Kavanagh, 2003; Hunter *et al*., 2007). Apoptosis is a cellular mechanism of self-destruction, involved in physiological as well as pathological processes and it can be either spontaneous or induced by exogenous agents among which are many drugs already used in cancer treatment.

The occurrence of an apoptotic pathway responsible for the activity of the polyenic compound **5** isolated from *E. pallida* was investigated in this study. The activity of caspases 3/7, which are executioners of both the intrinsic (or mitochondrial) and the extrinsic (or death receptor-related) apoptotic pathways (Hunter *et al*., 2007), as well as the presence of intracytoplasmatic internucleosomal fragments, was significantly enhanced after a 24 h time exposure with compound **5**, revealing that its cytotoxic effect is mediated through caspase-dependent apoptosis. The enzymatic assay used for the evaluation of DNA fragmentation, through the investigation on the extracellular compartment, let us verify the absence of necrotic cell death as a primary event involved in cytotoxicity induced by compound **5**.

The type of induced cell death was studied only in the early stages (24 h), as, *in vitro*, the absence of phagocytosis of cells in the late stages of apoptosis may induce them to undergo necrosis (apoptotic necrosis or secondary necrosis).

Compound **5** is likely to be able to have *in vivo* activity as it was shown in this study to be potentially bioavailable. It was one of three acetylenes in the *n*-hexane extracts from *E. pallida* that was able to cross the Caco-2 monolayers, which are an accepted model of intestinal absorption. The P_{app} of this and the other acetylenes indicates that they should be bioavailable in humans (Artursson and Karlsson, 1991). Total recovery of the acetylenes in the Caco-2 experiments was low (approximately 30%), suggesting that the acetylenes had been metabolized or retained in the cells of the monolayers. This is not unexpected as suicide inhibition of different enzymes by acetylenic structures has been previously reported (Bador and Paris, 1990).

In conclusion, our results demonstrate a direct cytotoxic effect of potentially bioavailable acetylenic compounds from *E. pallida* roots in two different cancer cell lines. The polyene compound **5**, the most potent constituent, revealed a pro-apoptotic mechanism of action and a good potential for absorption in humans when taken orally. These data encourage further investigation to clarify the exact mechanism of action of this polyene.

Conflict of interest

The authors state no conflict of interest.

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