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Cupressus lusitanica (Cupressaceae) leaf extract induces apoptosis in cancer cells

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Abstract

A crude ethanolic extract of *Cupressus lusitanica* Mill. leaves demonstrate cytotoxicity in a panel of cancer cell lines. Cell death was due to apoptosis, as assessed by morphologic features (chromatin condensation and apoptotic bodies formation) and specific DNA fragmentation detected by in situ end-labeling of DNA breaks (TUNEL). The apoptotic cell death was induced timely in a dose-dependent manner. Despite the absence of changes in the expression levels of antiapoptotic protein Bcl-2, proapoptotic Bax protein variants ω and δ were increased. These results warrant further research of possible antitumor compounds in this plant.
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Keywords: Antineoplasic drug; Bax variants; Bcl-2; *Cupressus lusitanica*; Apoptosis

1. Introduction

Apoptosis is a regulated cell death used by multicellular organisms to dispose unwanted cells. It is characterized by consecutive morphological events in which the chromatin condenses and becomes aggregated in sharply delineated masses, the cytoplasm shrinks and the plasmatic membrane suffers convolution (Kerr and Harmon, 1991). This type of death plays an important role in physiological processes and is altered in many pathological states. Recently, it has been shown that the mechanism of action of many antineoplasic drugs is based on apoptosis induction (Kerr and Harmon, 1991; Melendez-Zajgla et al., 1996; Maldonado et al., 1996). Drugs with antineoplasic activity have been isolated from plants, including taxoids and vinca alkaloids. The taxoids, one of the most clinically relevant groups of drugs, were isolated originally from the roots, stems and needles from three species of the genus *Taxus* (Taxaceae) (Schiff et al., 1979; Parness et al., 1982). Some ethnic groups in Mexico use empirically leaves of another Gymnosperm, Cupressaceae, *Cupressus lusitanica* Mill. for cancer treatment (Perez and Villavicencio, 1994).

In the present report we analyze the cytotoxic effect of a crude ethanolic extract of *C. lusitanica* in a panel of cancer cell lines.

2. Materials and methods

2.1. Cell culture

Non-small cell cancer cell line A549, cervicouterine cancer cell lines HeLa and CasKi, and hepatocarcinoma cell line HepG2 were maintained as a monolayer in Dulbecco's Modified Essential Medium (DMEM) containing 10% (v/v) fetal bovine serum at

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37 °C in a humidified atmosphere of a 5% (v/v) CO_2 in air. DMEM and fetal bovine serum were obtained from GIBCO BRL, Rockville, MD (USA); all other chemicals were obtained from Sigma, St. Louis (MO, USA), unless otherwise stated.

2.2. Ethanolic extract of C. lusitanica

C. lusitanica leaves were collected in the vicinity of Pachuca, Hidalgo (México). The material was identified by L. López and a voucher specimen (*Lopez # 13256*) was deposited in the 'Jorge Espinoza Salas' Herbarium of the Universidad Autonoma de Chapingo (UACh) for reference. The ethanolic extract was obtained by sequential extraction of 0.5 g of *C. lusitanica* at room temperature for 1 h with deionized water (5 ml) and ethanol (1 ml). The yield of the main peak between batches was $32.5 \pm 7.5\%$ of total extract (see Section 2.3). The supernatants were stored at −70 °C until use. Adjustments for concentration were performed with ethanol and subsequent experimental dilutions with deionized water.

2.3. HPLC profile

Procedures were performed as reported by Lee et al. (1999). The chromatographic system used was a Perkin–Elmer HPLC with diode array detector 235-C. Chromatographic separations were achieved using an inverse phase Waters Spherisorb ODS2 C18 column (250 × 4.6 mm, particle size of 5 μm) supplied by ABC Instrumentación Analítica (México, D.F.). The mobile phase consisting of acetonitrile: 0.1% phosphoric acid in deionized water (55:45 v/v) (Milli-Q Plus System, Molipore, Milford, MA, USA) was passed through a 0.22 μm membrane filter and subjected to chromatography at ambient temperature (21 °C). The flow-rate was maintained at 1.3 ml/min, with a detection wavelength of 230 nm. Peaks of the *Cupressus* extracts were compared to a paclitaxel standard (50 μl of a 15 μg/ml solution). Different lots were adjusted using the principal peak found at this absorbance (3.04 min in Fig. 1), in order to obtain uniform stocks for the toxicity assays. A representative chromatogram of the *Cupressus* extract is presented in Fig. 1. The retention time for the principal peak was 3.04 min. This peak was used to adjust the extracts in order to obtain normalized stocks for cytotoxic assays.

2.4. Cellular viability

Cells were seeded in 24-wells chamber dishes and exposed to several ethanolic extract dilutions of *C. lusitanica* for 1, 2 or 3 days. The cells were then fixed

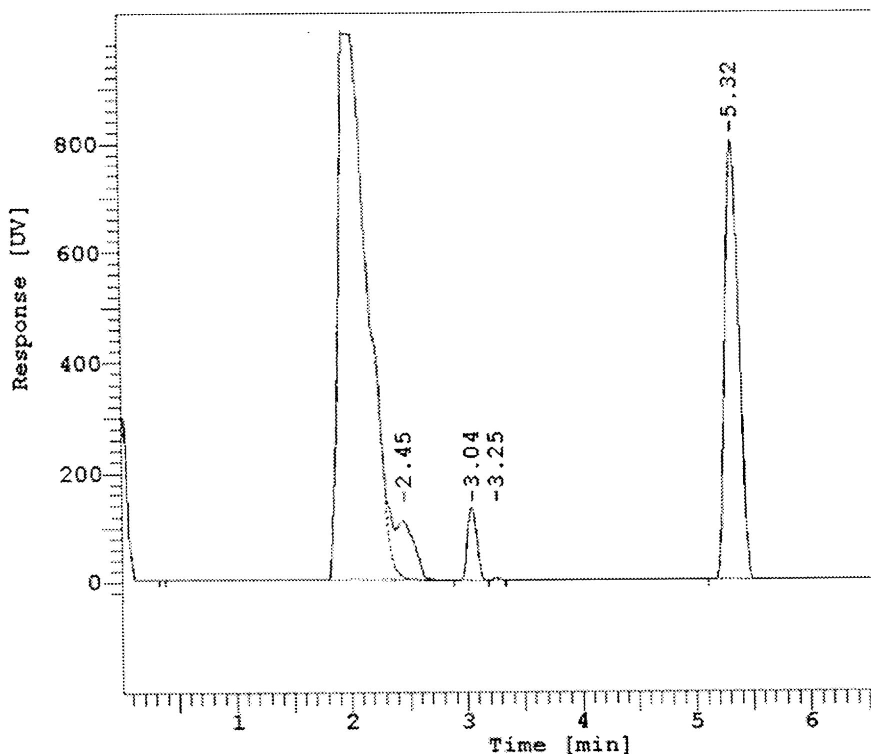

Fig. 1. Chromatogram of *C. lusitanica* extract spiked with paclitaxel. The detection wavelength was 230 nM. In this experiment the amount of 50 μl of a 1:200 dilution of the *C. lusitanica* extract was injected along with 50 μl of a 15 μg/ml dilution of paclitaxel. The main peak obtained presented a retention time of 3.04 min. Retention time for paclitaxel standard: 5.32 min.

with 70% ethanol at −20 °C for 15 min, washed in PBS and stained with crystal violet (1% in water). After washing, the stain was solubilized in 33% acetic acid and the absorbance determined in an ELISA reader at 570 nm. The analysis was performed in triplicate in four independent experiments.

2.5. Cytological examination

Cells were fixed with ethanol at −20 °C, pretreated with RNAse (10 μg/ml) for 30 min, stained for 5 min with ethidium bromide (10 μg/ml) and washed twice with PBS before mounting. The cells were then visualized with a Zeiss microscope, using epifluorescence and photographed in a Kodak Plus X-Pan film.

2.6. DNA fragmentation

DNA fragmentation was detected in situ by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) method (Gavrieli et al., 1992) with the in situ Cell Death Detection Kit (Roche Molecular Biochemicals) following the instructions of the manufacturer. Briefly: control and exposed cells were fixed with freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) for 1 h at room temperature. After washing twice with PBS the cells were permeabilized using a solution with 0.1% triton X-100 in 0.1% sodium citrate for 2 min on ice. The cells were then incubated for 60 min at 37 °C in TUNEL reaction containing TdT enzyme and nucleotide mixture in a reaction buffer. The cells were then washed three times with PBS and visualized under epifluorescence prior to being photographed as previously described.

2.7. Immunoblotting

Monolayer cultures were washed twice with ice-cold PBS. Extracts of HeLa cells exposed to a 1:5000 dilution of the crude ethanolic extract for 6, 12 and 24 h were prepared by lysis with RIPA buffer (1% NP40, 0.5% sodium deoxycholate and 0.1% SDS in PBS). Protein was quantified using a modified micro-Bradford procedure (Melendez-Zajgla et al., 1996). Equal amounts of protein were separated in a 10% SDS-PAGE, transferred to PVDF membranes (Amersham-Pharmacia, UK) and after blocking, incubated with monoclonal antibodies against Bcl-2 N-19 (Santa Cruz Biotechnology, CA, USA) and Bax Ab-1 (Amersham-Pharmacia, UK), washed and reincubated with anti-mouse or anti-rabbit IgG-HRP antibody (Amersham-Pharmacia, UK). Bax antibody is directed against aminoacids 150–165, and it recognizes five mRNA splice variants (α, β, γ, δ and ω) (Henkels and Turchi, 1999). The antibody binding was determined using enhanced chemiluminiscence (Roche Molecular Biochemicals), with X-Omat AR films (Kodak, Mexico) (Melendez-Zajgla et al., 1996).

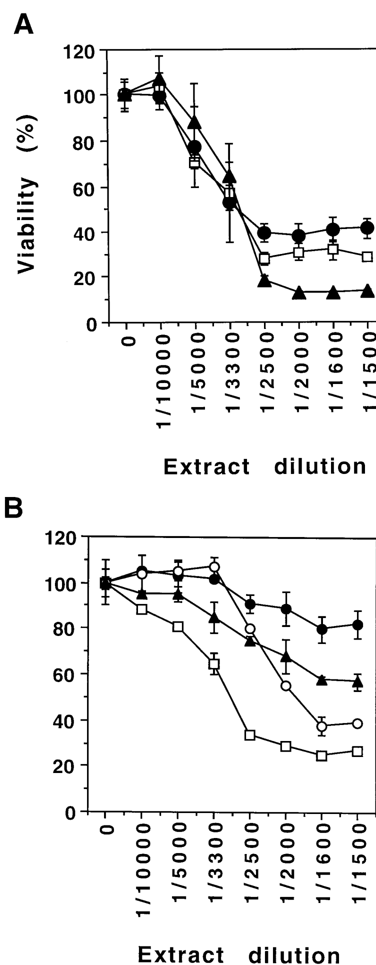

Fig. 2. (A) Viability of HeLa cells exposed to the ethanolic extract of *C. lusitanica* for 24 (●), 48 (□) or 72 (▲) h. (B) Viability of (●) A549, (□) HeLa, (○) Caski and (▲) HepG2 cells treated with the extract for 48 h. The viability is expressed as percentage from cells exposed to vehicle. The mean shown was calculated from three independent experiments performed by triplicate. The bars represent the standard deviation.

3. Results

In order to study the possible antineoplasic activity of *C. lusitanica* in vitro cytotoxic analysis in HeLa cells was performed. As shown in Fig. 2A, the extract produced a viability decrease dependent on the concentration and time of exposure. This effect was found with extract dilutions of 1:5000 at all times (24, 48 and 72 h). Maximal effect was produced with 1:2500 dilutions. The cytotoxicity was not restricted to this cell line, since CasKi and HepG2 cells were also sensible to the extract (Fig. 2B), although A549 cells were resistant at the time of analysis. Longer exposure to the extract was required for cytotoxicity effect in these cells (results not shown).

To investigate if the viability decrease was due to a specific death type, nuclear morphology of exposed and vehicle-treated cells was analyzed with ethidium bromide. As shown in Fig. 3, nuclei of HeLa cells exposed to the extract presented condensed and fragmented chromatin, in clear contrast to the spherical and regular form of the control nuclei. These changes are suggestive of apoptosis, as described elsewhere (Imreh et al., 1998). In order to support this finding we detected specific DNA fragmentation in situ using the TUNEL technique, a widely used assay to detect apoptotic cells (Gavrieli et al., 1992). Fig. 4 shows positive staining in cells exposed for 24 h to the extract, whereas in control cells there was no staining. Similar results were obtained in CasKi and HepG2 cells (results not shown).

Finally, we analyzed the steady-state levels of two proteins involved in the apoptotic pathway in several experimental models, Bcl-2 and Bax. Fig. 5 shows a representative blot of a time course in HeLa cells exposed to the extract. The levels of Bcl-2 protein and the principal variant of Bax in these cells (alpha) remained constant. Nevertheless, 26 and 16 kDa Bax variants (δ and ω) were expressed de novo during the exposure to *C. lusitanica* extract, with the highest levels achieved just previous to the apoptosis onset.

4. Discussion and conclusion

At least 121 chemical substances useful as drugs are still isolated from plants throughout the world (Farnsworth et al., 1985). Some of the most useful antineoplasic drugs have been extracted also from plants. Among them, Paclitaxel (Taxol), isolated from three species of the genus *Taxus* (Taxaceae) (Parness et al., 1982; Schiff et al., 1979), has particular importance in the treatment of a variety of solid tumors. In the present work we analyzed the in vitro effect on tumor cells of the ethanolic extract of *C. lusitanica*. In Mexico, this plant is used by the Nahuas of the Huasteca region as a medicine to treat cancer (Perez and Villavicencio, 1994). Although an aqueous extract produced no effect in HeLa cells (data not shown), the ethanolic extract proved to be cytotoxic in a time- and concentration-dependent manner. This cytotoxic response was due to apoptosis, as corroborated with morphologic analysis and DNA fragmentation assays.

Members of the Bcl-2 family of proteins interact to regulate apoptosis (Adams and Cory, 1998). Homodimers and heterodimers formed by proteins of this family can either promote or inhibit apoptosis (Elliott et al., 1999). The balance between protein levels of these members is crucial for the cellular decision of starting the apoptotic process. Proapoptotic members of this family include Bax (bcl-2-associated protein X) protein, and apoptotic inhibitors that include Bcl-2 protein (Cosulich et al., 1997; Jean et al., 1999). In a first attempt to investigate the pathway(s) responsible for the *C. lusitanica* effect, we analyzed Bcl-2 and Bax levels during exposure to the extract. Although Bax α remained constant, variants ω and δ were detected previous to the onset of apoptosis. This de novo expression is relevant, since the induction of Bax has been implicated in the initiation of chemotherapy-induced apoptosis (Henkels and Turchi, 1999; Oltval et al., 1993; Jurgensmeier et al., 1998). In particular, it has been shown that transient overexpression of Bax ω protein potentiates cell death at levels comparable to that of Bax-α overexpression (Zhou et al., 1998). In the present experimental model, Bcl-2 levels were not modified, in contrast to the apoptosis induced by cisplatin in HeLa cells, in which Bcl-2 protein decreases prior to cell death (Maldonado et al., 1997). This difference could be indicative of alternative regulation of these proteins and/or perhaps, a different apoptotic pathway.

Medicinal natural resources may contribute to pharmaceutical and health services, since they may be used

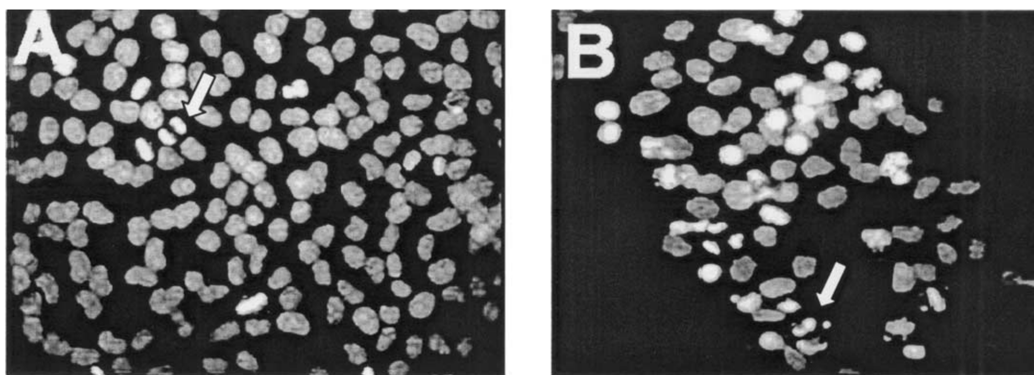

Fig. 3. Nuclear morphology of HeLa cells exposed to vehicle (A) or to *C. lusitanica* extract (B) (1:5000 for 24 h). The nuclei were stained with Ethidium Bromide. The white arrow in panel A shows a mitotic cell and in panel B an apoptotic nucleus. Note the apoptotic bodies in the extract-exposed cells. Original magnification ×40. These are representative results from four independent experiments.

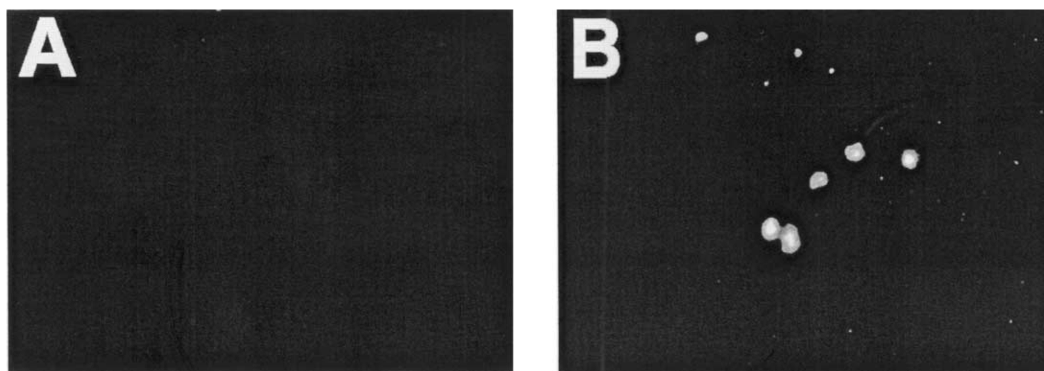

Fig. 4. DNA fragmentation of HeLa cells exposed to vehicle (A) or to *C. lusitanica* extract (1:5000 for 24 h) (B) detected by TUNEL technique. Original magnification ×40.

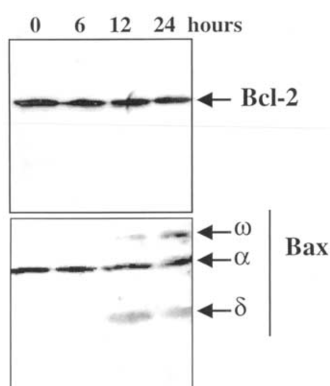

Fig. 5. Steady-state level of Bcl-2 (upper panel) and Bax proteins (lower panel) by western blot analysis. HeLa cells exposed to a 1:5000 dilution of the *C. lusitanica* extract for the times shown. This is a representative blot from four independent experiments.

directly as pharmaceuticals, as templates for chemical synthesis of related medicinal compounds or as investigative, evaluative or research tools in drug development. We are now in the process of isolating the active compound(s) responsible for the results presented here. Although not showed in the present research, the HPLC profile obtained points toward the existence of related taxoids in the extract. More research concerning the possible utility of *C. lusitanica* in cancer treatment is warranted.

References

Adams, J.M., Cory, S., 1998. The Bcl-2 protein family: arbiters of cell survival. Science 281, 1322–1326.

Cosulich, S.C., Worrall, V., Hedge, P.J., Green, S., Clarke, P.R., 1997. Regulation of apoptosis by BH3 domains in a cell-free system. Current Biology 7, 913–920.

Elliott, M.J., Murphy, K.M., Stribinskiene, L., Ranganathan, V., Sturges, E., Farnsworth, M.L., Lock, R.B., 1999. Bcl-2 inhibits early apoptotic events and reveals post-mitotic multinucleation without affecting cell cycle arrest in human epithelial tumor cells exposed to etoposide. Cancer Chemotheraupeutic Pharmacology 44, 1–11.

Farnsworth, N.R., Akerele, O., Bingel, A.S., Soejarto, D.D., Guo, Z., 1985. Medicinal plants in therapy. Bulletin of the World Health Organization 63, 965–981.

Gavrieli, Y., Sherman, Y., Ben-Sasson, S.A., 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. The Journal of Cell Biology 119, 493–501.

Henkels, K.M., Turchi, J.J., 1999. Cisplatin-induced apoptosis proceeds by Caspase-3-dependent and independent pathways in Cisplatin-resistant and -sensitive human ovarian cancer cell lines. Cancer Research 59, 3077–3083.

Imreh, G., Beckman, M., Iverfeldt, K., Hallberg, E., 1998. Noninvasive monitoring of apoptosis versus necrosis in a neuroblastoma cell line expressing a nuclear pore protein tagged with the green fluorescent protein. Experimental Cell Research 238, 371–376.

Jean, J.C., Oakes, S.M., Joyce-Brady, M., 1999. The Bax inhibitor-1 gene is differentially regulated in adult testis and developing lung by two alternative TATA-less promoters. Genomics 57, 201–208.

Jurgensmeier, J.M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D., Reed, J.C., 1998. Bax directly induces release of Cytochrome C from isolated mitochondria. Proceedings of the National Academy of Science USA 95, 4997–5002.

Kerr, J.F.R., Harmon, B.V., 1991. Definition and incidence of apoptosis: an historical perspective. In: Tomei, L.D., Cope, F.O. (Eds.), Current Communications in Cell and Molecular Biology: Apoptosis: The Molecular Basis of Cell Death, vol. 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 5–13.

Lee, S.-H., Yoo, S.D., Lee, K.-H., 1999. Rapid and sensitive determination of paclitaxel in mouse plasma by high-performance liquid chromatography. Journal of Chromatography B 724, 357–363.

Maldonado, V., De Anda, J., Melendez-Zajgla, J., 1996. Paclitaxel-induced apoptosis in HeLa cells is serum-dependent. Journal of Biochemical Toxicology 11, 546–556.

Maldonado, V., Mélendez, J., Ortega, A., 1997. Modulation of NF-kB, p53 and Bcl-2 in the apoptotic process induced with cisplatin in HeLa cells. Mutation Research 381, 67–75.

Melendez-Zajgla, J., García, C., Maldonado, V., 1996. Subcellular redistribution of Hsp 72 protein during cisplatin-induced apoptosis in HeLa cells. Biochemistry and Molecular Biology International 40, 253–261.

Oltval, Z.N., Milliman, C.L., Korsmeyer, S.J., 1993. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax that accelerates programed cell death. Cell 74, 609–619.

Parness, J.E., Kingston, D.E., Powell, R.D., Harracksinght, S., Horwitz, S., 1982. Structure-activity study of cytotoxicity and microtubule assembly *in vitro* by taxol and related taxanes. Biochemical and Biophysical Research Communications 105, 1082–1089.

Perez, E.B.E., Villavicencio, M.A., 1994. Listado de las Plantas Medicinales del Estado de Hidalgo. Universidad Autonoma del Estado de Hidalgo Press, 10.
Schiff, P.B., Fant, J.P., Horwitz, S.B., 1979. Promotion of microtubule assembly *in vitro* by Taxol. Nature 277, 665–667.
Zhou, M., Demo, S.D., McClure, T.N., Crea, R., Bitler, C.M., 1998. A novel splice variant of the cell death-promoting protein BAX. Journal of Biological Chemistry 273, 11 930–11 936.