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Potential Anticancer Activity Against Human Epithelial Cancer Cells of *Peumus boldus* Leaf Extract

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The potential *in vitro* antineoplastic effect has been studied of a methanolic extract of leaves of *Peumus boldus* Molina (Monimiaceae) on two human cancer epithelial cell lines, DU-145 cells (androgen-insensitive prostate cancer cells) and KB cells (oral squamous carcinoma cells). Our findings show that this extract exhibited comparable effects on the cancer cells examined as judged by IC_{50} values (5.07±0.4 μg/mL and 5.28±0.5 μg/mL in DU-145 and KB cells, respectively). In addition, with respect to genomic DNA damage, determined by Comet assay, the results obtained show a high fragmentation of DNA, not correlated to lactic dehydrogenase (LDH) release, a marker of membrane breakdown, in both cell lines treated with the extract at 5-20 μg/mL concentrations. Taken together, our experimental evidence may justify further investigation of the chemopreventive and chemotherapeutic potential of this natural drug.

Keywords: *Peumus boldus* Molina, DU-145 cells, KB cells, cell growth, DNA fragmentation, LDH release.

Boldo consists of the dried leaf of *Peumus boldus* Molina (Monimiaceae), an evergreen shrub or a small tree growing from central and southern Chile. Boldo, used traditionally in South America mainly against liver diseases, is recognized as a herbal remedy in a number of Pharmacopeias, and is employed in the form of infusions, tinctures and extracts [1,2]. Boldo leaf contains different alkaloids belonging to the large benzylisoquinoline-derived family. Boldine [(*S*)-2,9-dihydroxy-1,10-dimethoxy-aporphine], the main aporphine alkaloid in boldo leaves and barks, seems to be particularly important as a natural antioxidant [1,2].

Leaves of *P. boldus* contain also essential oils of complex and variable composition, tannins and flavonoids, such as flavonol glycosides, kaempferol, quercetin and catechin. This last compound is the flavonoid that is most abundant and with the alkaloid boldine is the main contributor of the antioxidant activity of Boldo leaf extracts [3,4].

Cancer is the largest single cause of death in both men and women. Recently, resistance to anticancer drugs has been observed. Therefore, the research and development of more effective and less toxic drugs by the pharmaceutical industry has become necessary. Many substances present in plants, and in particular the flavonoids, are known to be effective and versatile chemopreventive and antitumor agents in a number of experimental models of carcinogenesis [5-8]. In view of these considerations, Boldo leaf could possess anticancer activity and, therefore, could be useful in the prevention or treatment of cancer. The present study was undertaken to investigate the *in vitro* cytotoxic effect of a methanolic extract from leaves of *P. boldus* on two human epithelial cancer cell lines, DU-145 cells (androgen-insensitive prostate cancer cells) and KB cells (oral squamous carcinoma cells).

To evaluate the effect of the extract from *P. boldus* leaves on cell growth of human cancer cells, we

Table 1: Cell growth inhibition, assayed using MTT test, of DU-145 and KB cells untreated and treated with methanolic extract from *P. boldus* leaves at different concentrations for 72 h.

Treatments	DU-145 cells	KB cells
	IC_{50} [a] (μg/mL)	
P. boldus extract	5.07±0.4	5.28±0.5
Doxorubicin	9.37±1.1	1.43±0.7

[a]Results are expressed as IC_{50} values (μg/mL) ± SD. The IC_{50} value, relative to untreated control, represents the concentration that inhibited cell vitality by 50%. Doxorubicin was used as a positive control. Each value represents the mean ± SD of three experiments, performed in quadruplicate.

cultured the cells in either the absence or presence of this natural product. After treatment for 72 h, the MTT assay, a non-radioactive assay widely used to quantify cell viability and proliferation, was performed. The results, summarized in Table 1, show that our extract was active and exhibited comparable degrees of antigrowth effect in both cancer cells examined as judged by IC_{50} values, 5.07±0.4 μg/mL and 5.28±0.5 μg/mL in DU-145 and KB cells, respectively.

Necrosis results in a disruption of the cytoplasmic membrane and the necrotic cells release cytoplasmic lactic dehydrogenase (LDH) and other cytotoxic substances into the medium. We therefore, in a next series of experiments, examined the membrane permeability of treated cells by the existence of LDH in their culture medium. No increase in LDH release was observed in these cancer cells treated with the methanolic extract of *P. boldus* leaves at 5-10-20 μg/mL concentrations. Conversely, a significant increase ($p<0.001$) in LDH was observed at 40 μg/mL (Figure 1).

Nuclear DNA was analyzed using single-cell gel electrophoresis (SCGE), known as the Comet assay, a sensitive method for the visualization of DNA damage measured at the level of individual cells and a versatile tool that is highly efficacious in human bio-monitoring of natural compounds.

The Comet assay also allows us to distinguish apoptotic from normal and necrotic cells based on the DNA fragmentation pattern [9]. The Comet pattern significantly differs between apoptotic and control cultures, as well as between apoptotic and necrotic cultures. Quantification of the Comet data, in our experimental conditions is reported as TDNA and TMOM in Table 2. The results clearly display DNA damage in cells exposed to the *P. boldus* leaf extract

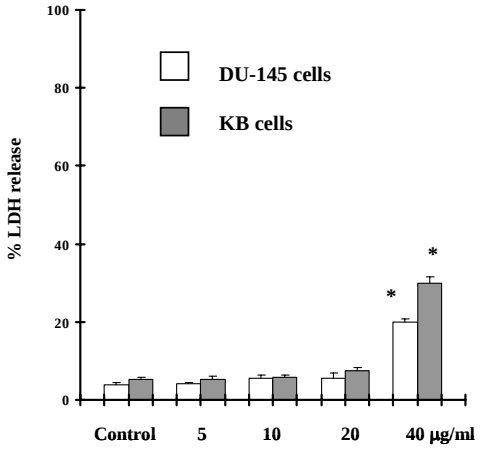

Figure 1: Lactate dehydrogenase (LDH) release, expressed as percentage of LDH, released into the cell medium with respect to total LDH, in DU-145 and KB cells treated with methanolic extract of *P. boldus* leaves at different concentrations for 72 h. Each value represents the mean ± SD of three experiments, performed in quadruplicate. *Significant vs. control untreated cells ($p<0.001$).

Table 2: Comet assay of genomic DNA of DU-145 and KB cells untreated and treated with methanolic extract of *P. boldus* leaves at different concentrations for 72 h.

Treatments	TDNA	TMOM
DU-145 cells		
Control	15.7±3.0	88±3.7
5μg/mL	99±4.0*	975±15*
10 μg/mL	149±6.0*	1035±12*
20 μg/mL	179±4.3*	1645±43*
40μg/mL	30±5.5°	287±10*
KB cells		
Control	51±4.0	175±5.4
5μg/mL	205±5.0*	1498±16*
10 μg/mL	298±6.8*	2919±14*
20 μg/mL	259±3.0*	2807±13*
40μg/mL	84±5.9*	337±17*

Each value represents the mean ± SD of three experiments, performed in quadruplicate. *Significant vs. control untreated cells ($p<0.001$).

after 72 h, with a drastic increase in both TDNA and TMOM at 5-10-20 μg/mL concentrations.

These findings suggest that this extract induces, in our experimental conditions, apoptotic cell death, in accordance with the literature data, which indicate that only comets with high values of TMOM (tail moments) and TD (distance between head and tail of the comet) can be related to apoptosis [10]. Alternatively, in cells exposed to the extract at 40 μg/mL concentration for 72 h, the Comet assay did not show typical comet–like structures that occur during apoptosis.

The extract used in the present work could contain boldine, catechin, tannins and other flavonoids and

alkaloids, according to reported data [1]. Nevertheless, it not possible from this study to attribute the activity to any of them. However, some suppositions can be advanced on the base of literature data. Flavonoids, kaempferol, quercetin and catechin have been shown to exhibit anticancer activities in preclinical studies [6-8]. The recent studies of Hoet *et al*. [11] showed that boldine was inactive in a cell growth inhibition assay using HeLa cells (human epithelial cancer cell line from cervical carcinoma).

Taken together, our experimental data may justify further investigation of the chemopreventive and chemotherapeutic potential of this natural drug.

Experimental

Chemicals: All reagents were of commercial quality and were used as received. Boldine, catechin, 3(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) and β-nicotinamide-adenine dinucleotide (NADH) were obtained from Sigma Aldrich Co (St. Louis, USA). All other chemicals were purchased from Sigma Aldrich Co (St. Louis, USA) and GIBCO BRL Life Technologies (Grand Island, NY, USA).

Plant material and extraction: The leaves of *P. boldo* were collected at Quintay (Valparaiso) in January 2006. A voucher specimen (voucher specimen #12-07) was deposited in the Department of Chemistry, Universidad Santa Maria, Valparaiso, Chile. The leaves were exhaustively extracted with methanol and concentrated under vacuum to give a residue [yield 165g (14.3%)].

Cell culture and treatments: DU-145 cells (androgen-insensitive prostate cancer cells) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM glutamine, and 1% non essential amino-acids. KB cells (oral squamous carcinoma cells) were maintained in RPMI supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were plated at a constant density to obtain identical experimental conditions for the different tests, thus achieving a high accuracy of the measurements. After 24 h incubation at 37°C under a humidified 5% carbon dioxide atmosphere to allow cell attachment, the cells were treated with different concentrations of methanolic extract of *P. boldus* leaves, and incubated for 72 h under the same conditions. Doxorubicin was used as a positive control. Stock solution of the tested compound was prepared in ethanol and the final concentration of this solvent was kept constant at 0.25%. Control cultures received ethanol alone.

MTT bioassay: Cellular growth was determined using the MTT assay on 96-well microplates, as previously described [12]. The optical density of each well sample was measured with a microplate spectrophotometer reader (Digital and Analog Systems, Rome, Italy) at 550 nm.

Lactic dehydrogenase (LDH) release: Lactic dehydrogenase (LDH) activity was spectrophotometrically measured in the culture medium and in the cellular lysates at 340 nm by analyzing NADH reduction during the pyruvate-lactate transformation, as previously reported [12]. The percentage of LDH released was calculated as the percentage of the total amount, considered as the sum of the enzymatic activity present in the cellular lysate and that in the culture medium. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used.

DNA analysis by Comet assay: The presence of DNA fragmentation was examined by single cell gel electrophoresis (Comet assay), as previously reported [12]. At the end of the electrophoretic run, the "minigels" were neutralized in 0.4 M Tris-HCl, pH 7.5, stained with 100 µL of ethidium bromide (2 µg/mL) for 10 min and scored using a fluorescence microscope (Leica, Wetzlar, Germany) interfaced with a computer. Software (Leica-QWIN) allowed us to analyze and quantify DNA damage by measuring: a) tail length (TL), intensity (TI) and area (TA); b) head length (HL), intensity (HI) and area (HA). These parameters are employed by the software to determine the level of DNA damage as: i) the percentage of the fragmented DNA (TDNA), and ii) tail moment (TMOM) expressed as the product of TD (distance between head and tail) and TDNA.

Statistical analysis: Statistical analysis of results was performed by using one-way ANOVA followed by Dunnett's post-hoc test for multiple comparisons with control. All statistical analyses were performed using the statistical software package SYSTAT, version 9 (Systat Inc., Evanston IL, USA). Each value represents the mean ± SD of three separate experiments performed in quadruplicate.

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