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Acetylcholinesterase inhibition, antioxidant activity and toxicity of *Peumus boldus* water extracts on HeLa and Caco-2 cell lines

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ABSTRACT

This work aimed to study the inhibition on acetylcholinesterase activity (AChE), the antioxidant activity and the toxicity towards Caco-2 and HeLa cells of aqueous extracts of *Peumus Boldus*. An IC_{50} value of 0.93 mg/mL, for AChE inhibition, and EC_{50} of 18.7 μg/mL, for the antioxidant activity, was determined. This activity can be attributed to glycosylated flavonoid derivatives detected, which were the main compounds, although boldine and other aporphine derivatives were also present. No changes in the chemical composition or the biochemical activities were found after gastrointestinal digestion. Toxicity of *P. boldus* decoction gave an IC_{50} value 0.66 mg/mL for HeLa cells, which caused significant changes in the cell proteome profile.

1. Introduction

Peumus boldus (boldo) is a native plant from Chile, belonging to the Monimiaceae family. Infusions of leaves of this herb are recommended to treat digestive and hepatic problems (Ruiz et al., 2008). To treat some digestive problems and accelerate colonic transit, inhibitors of acetylcholinesterase are used (Jarvie et al., 2008; Law et al., 2001). These inhibitors increase the motility helping to accelerate the digestive process.

Due to the consumption of the infusion or decoction from plants, the action of enzymes like pepsin and pancreatin, from the gastrointestinal tract, on the biochemical transformation of the extracts constituents must be known. There are reports in which the biological activity evaluated in plant extracts was diminished after the digestive process (Cilla et al., 2009; Porfírio et al., 2010). These effects depend on the type of compounds present in the infusions. It is necessary to have some insight into the digestibility of the herbal infusions that are consumed.

The effects ascribed to *P. boldus*, some of them toxic, have occasionally been attributed to the presence of the alkaloid boldine. Indeed, there are several studies analyzing the biochemical activity of this alkaloid (Ruiz et al., 2008). Some restrictions to the use of this plant must be considered due to its toxic effects, mainly in pregnant women (Almeida et al., 2000). In aqueous extracts of *P. boldus* the principal components were found to be polyphenols and boldine was not detected (Simirgiotis and Schmeda-Hirschmann, 2010). This alkaloid is mainly present in the volatile fraction (Simirgiotis and Schmeda-Hirschmann, 2010) or in the hidro-alchoolic extracts of the plant (Almeida et al., 2000).

There are some reports on the toxicity of *P. boldus,* although these studies are mainly for the use of dry leaves and when boldine is used as a pure chemical compound. Indeed, very few data on infusions or decoctions of this plant leaves exist (Simirgiotis and Schmeda-Hirschmann, 2010).

The toxicity of the water extracts from these plants can be evaluated on culture cell lines. Caco-2 cells (Human epithelial colorectal adenocarcinoma cells) are usually used to simulate the intestinal digestive tract. When grown under certain experimental conditions, they differentiate into enterocyte-like cells (Hidalgo and Li, 1996). HeLa cells (cervix epithelial adenocarcinoma) are also used to perform this type of studies and several reports can be found in the literature, with which the results can be compared to.

As most of the effects reported for boldo are attributed to the boldine presence, which is usually not found in the water extracts, Simirgiotis and Schmeda-Hirschmann (2010), it is important to determine the biochemical activities of *P. bolduś* herbal tea. The

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main objective of the present work is to evaluate the effect of aqueous boldo extracts on the inhibition of acetylcholinesterase, their antioxidant activity and the effect of these extracts on the total protein expression in the cell. These results may help to explain some of the ethno-botanic uses described for this herb and to predict some careful applications with the infusions uses for the general population.

2. Materials and methods

2.1. Plant material

P. boldus was acquired from a national producer of herbal teas in the dry form and already packed and sold in supermarkets and pharmacies.

2.2. Chemicals

All chemicals were of analytical grade. Acetylcholinesterase (AChE) type VI-S, from electric eel 349 U/mg solid, 411 U/mg protein, 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), acetylthiocholineiodide (AChI), dimethylsulphoxide (DMSO), β-glucuronidase type IX-A from *Escherichia coli* 1134600, 3660000 U/g protein, DPPH(2,2-di (4-tert-octylphenyl-1-picrylhydrazyl), HEPES buffer, pancreatin, MTT (thiazolyl tetrazolium bromide), polyethylene glycol, acetic acid was acquired from Fluka. DMEM (Dulbecco's Modified Eagle Medium), HBSS (Hank's Balanced Salt Solution), glutamine, Pen–Strep (penicillin–streptomycin), PBS (phosphate buffered saline), FBS (fetal bovine serum) and trypsin were bought from Lonza, Verviers, Belgium. Methanol and acetonitrile, both HPLC grade, were obtained from Merck (Darmstadt, Germany). CHAPS (3-(3-cholamidopropyl)-dimethyl ammonio)-1-propanesulfonate, urea, DTT, iodoacetamide, from Sigma. Bradford reagent from Farbstoff-Konzentrat. Molecular weight markers from BioRad. IPG buffer for pH 3-11 NL, 7-cm IPG Immobiline DryStrips pH 3-11 NL, DryStrip cover fluid and Kit PlusOne silver staining were from GE-Healthcare.

2.3. Extract preparation

Aqueous extract of *P. boldus* was prepared as a decoction, 10 g of ground fresh leaves boiled for 10 min at 100 °C, in 300 mL of distilled water and filtered through 1 grade Whatman paper. To prepare infusions, the same amount was suspended for 10 min in water that had stopped boiling. The infusion was also filtered and both extracts were lyophilized. The yield of extraction was approximately116 mg of extract/g of plant and 19 mg of extract/g of plant in the decoction and infusion process, respectively.

2.4. HPLC-DAD analysis

The HPLC analysis was carried using a Liquid Chromatograph Finnigan™ Surveyor-Plus Modular LC System, Thermo-Finnigan (Waltham, MA) equipped with a Purospher-STAR RP-18 column, from Merck (Darmstadt, Germany) and Xcalibur software. The extracts were analysed by HPLC, injecting 25 μL of 0.1 mg/ml extract with an auto injector, and using a linear gradient composed of solution A (0.05% trifluoroacetic acid), solution B (acetonitrile) and solution C (methanol) as follows: 0 min, 90% A, 2% B, 8% C; 15 min 70% A, 2% B, 28% C; 18 min 60%A, 5% B, 35% C; 30 min 50% A, 10% B, 40% C; 34 min 90% A, 2% B, 8% C. The solutions containing 100 mM of several standards in distilled water were also injected. The detection was carried out between 200 and 600 nm with a diode array detector.

2.5. LC–MS and LC–MS/MS analysis

The LC–MS and LC–MS/MS analysis were carried out on a liquid chromatograph Surveyor Plus Modular LC system connected to a LCQ Duo ion trap mass spectrometer equipped with an electrospray ionisation (ESI) source, from Thermo Scientific (Bremen, Germany). The column used was a Luna® 5 μm C8 100 Å HPLC column from Phenomenex (Madrid, Spain). The extracts were analysed by injection of 25 μL and using a linear gradient composed of solution A (1.0% formic acid), solution B (acetonitrile) and solution C (methanol) as follows: 0 min, 90% A, 2% B, 8% C; 15 min 70% A, 2% B, 28% C; 18 min 60% A, 5% B, 35% C; 30 min 50% A, 10% B, 40% C; 34 min 90% A, 2% B, 8% C at a flow rate of 1 mL min^{-1}. The mass spectrometer was operated in both positive and negative ion modes in the range *m/z* 120–1000 and the parameters were adjusted in order to optimize the signal-to-noise ratios (S/N) for the ions of interest. Briefly, the nebulizing and auxiliary gas (nitrogen) flow rates were 40 and 20 (arbitrary units) and the capillary temperature was set to 250 °C. Collision induced dissociation (CID) experiments were performed by isolating the ions within the ion trap and accelerating them in order to suffer multiple collisions with the background gas present in the ion trap (helium) using a data dependent acquisition mode. The ions of interest were activated by applying a percentage of a supplementary a.c. potential in the range of 0.75–1.75 V_{p-p} (peak-to-peak) to the end cap electrodes of the ion trap at the resonance frequency of the selected ion (referred to as the Normalized Collision Energy, NCE). The injection times were 50 ms in a full scan and 200 ms in an MS/MS scan. Xcalibur™ software from Thermo Scientific was used to acquire and process the data.

Most of the compounds present in the extract studied were identified by mass spectral database search using the MassBank database (Horai et al., 2010).

2.6. Acetylcholinesterase inhibition

Acetylcholinesterase enzymatic activity was measured using the method described by Ingkaninan et al. (2003), with the adaptation previously reported Porfírio et al. (2010). The results are the mean of 3 replicates.

2.7. Determination of antioxidant activity

Antioxidant activity was measured by the DPPH method (Tepe et al., 2005), with a slight modification described in Porfírio et al. (2010). The results are the mean of 3 replicates.

2.8. In vitro metabolism by the gastric and pancreatic juices

The assay was adapted from Yamamoto et al. (1999) and published previously in Porfírio et al. (2010). The results are the mean of 5 replicates.

2.9. Antiproliferative studies

Caco-2 cells (ATCC#HTB-37) and HeLA cells (HeLa (ATCC#CCL-2) were cultured in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin, 100 U/mL streptomycin, and 2 mM L-glutamine, at 37 °C in an atmosphere with 5% CO_2. The culture medium was changed every 48–72 h and the cells were tripsinized when confluence reached approximately 80%.

To study the antiproliferative effect of the plant extracts the cells were seeded in 96 microplates, using 5×10^4 cells/100 μL in each well. Cells were grown for 120 h (Caco-2) or 48 h (HeLa). Each culture medium was replaced by DMEM containing different concentrations of *P. boldus* decoction. Cells were incubated for 4 h at 37 °C in with 5% CO_2. The quantification of the antiproliferative effect was carried out using the MTT method described in Chen et al. (2008). The absorbance of each well was compared to the control cells, without *P. boldus* extract. IC_{50} represents the extract concentration that causes 50% decrease in cell viability. This value was calculated from a regression curve in which the cell viability was represented versus the extract concentration in each well. The values are the mean of 4 times 8 replicates.

2.10. 2D gel electrophoresis

2D-PAGE was carried out in HeLa cells subject to the IC_{50} value of *P. boldus* decoction. Cells were grown for 4 h at 37 °C with plant extract and a control was grown in the absence of the extract. Cells were then washed three times with PBS buffer pH 7.5. After washing, 500 μL of lysis buffer (4% (w/v) CHAPS, 8 M urea) was added to each plate (containing $\sim 1 \times 10^7$ cells) and cells were scraped from the plate (always on ice). The suspension was centrifuged at 10000*g* at 4 °C for 10 min. The soluble fraction (fraction 2) was recovered. The pellet was suspended in 100 μL of 7 M urea, 2 M thiourea and 4% (w/v) CHAPS (adapted from Fountoulakis et al., 2004) and this fraction was called insoluble fraction (fraction 1). Protein content was quantified using the Bio-Rad Protein Assay reagent. For 2D-PAGE, 66 μg of protein were solubilized in 125 μL of lysis buffer containing 0.002% (w/v) bromophenol blue, 1% (w/v) DTT and 0.5% IPG buffer at pH 3-11 NL, for one hour under agitation. The sample was applied to a 7 cm Immobiline DryStrip pH 3-11 NL and isoelectric focusing was performed at an Ettan™ IPGphor II™ (Amersham). Active in-gel sample rehydration was performed at 30 V over 12 h. Proteins were focused using six phases of stepped voltages from 150 to 8000 V. Focused strips were incubated in equilibrium buffer (6 M urea, 75 mM Tris pH 8.8, 29.3% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue) with 1% (w/v) DTT for 15 min at room temperature with gentle agitation. Strips were then transferred to equilibrium buffer containing 2.5% (w/v) iodoacetamide. The second dimension was performed in a 12% polyacrylamide gel and 2D gels were silver stained using the PlusOne silver staining kit. Differentially expressed proteins were identified by visual inspection after digitalization.

2.11. Statistical analysis

All results are presented as mean ± standard deviation of five replicates and the software used was Microsoft Excel-2002.

3. Results and discussion

3.1. Main composition of P. boldus

The herbal tea of *P. boldus*, either as infusion or decoction was analysed by HPLC-DAD (Fig. 1a) and by LC-MS (Fig. 1b and c).