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Research Paper

Boldo prevents UV light and nitric oxide-mediated plasmid DNA damage and reduces the expression of Hsp70 protein in melanoma cancer cells

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Abstract

Objectives This study was designed to investigate the potential protective effect of a methanolic extract of *Peumus boldus* leaves on UV light and nitric oxide (NO)-mediated DNA damage. In addition, we investigated the growth inhibitory activity of this natural product against human melanoma cells (M14).

Methods Boldine, catechin, quercetin and rutin were identified using a HPLC method. The extract was incubated with plasmid DNA and, before irradiating the samples with UV-R, H_2O_2 was added. For analysis of DNA single-strand breaks induced by NO, the experiments were performed by incubating the extract with Angeli's salt. In the study on M14 cell line, cell viability was measured using MTT assay. Release of lactate dehydrogenase, a marker of membrane breakdown, was also measured. For the detection of apoptosis, the evaluation of DNA fragmentation (COMET assay) and caspase-3 activity assay were employed. The expression of heat shock protein 70 (Hsp70) was detected by Western blot analysis. Generation of reactive oxygen species was measured by using a fluorescent probe.

Key findings The extract (demonstrating the synergistic effect of the constituents boldine and flavonoids), showed a protective effect on plasmid DNA and selectively inhibited the growth of melanoma cells. But a novel finding was that apoptosis evoked by this natural product in M14 cells, appears to be mediated, at least in part, via the inhibition of Hsp70 expression, which may be correlated with a modulation of redox-sensitive mechanisms.

Conclusions These results confirm the promising biological properties of *Peumus boldus* and encourage in-vivo investigations into its potential anti-cancer activity.

Keywords apoptosis; heat shock protein; melanoma cells; *Peumus boldus*; ultraviolet radiation

Introduction

Boldo consists of the dried leaf of *Peumus boldus* Molina (Monimiaceae), an evergreen shrub or a small tree growing in central and southern Chile. It has been used for its medicinal properties by diverse indigenous groups, including the Mapuche ethnia who lived in Chile before the arrival of the Spanish in the fifteenth century.[1] Actually, boldo is widely used in Chilean folk medicine and is recognised as a medicinal herb in Pharmacopoeia.[1] Introduced in France in about 1870 by Bourgoin and Verne, this species is described in the French Pharmacopoeia. It is also included in the Pharmacopoeias of Switzerland, Germany, Brazil, Chile, Portugal, Rumania and Spain and it is employed in the form of infusions, tinctures and extracts.[1,2] Its choleretic and cholagogue properties are often reported. Besides these main indications, boldo is also used as a diuretic, urinary tract anti-inflammatory agent, stomachic, sedative and emetic, and in the treatment of headache, earache, toothache and rheumatism.[2] Boldo leaves contain 0.4–0.5% of at least 17 different alkaloids belonging to the large benzylisoquinoline-derived family. Boldine is the major alkaloid and its content in boldo leaves is 0.12%.[3] Leaves of *P. boldus* also contain essential oils of complex and variable composition, tannins and flavonoids, such as catechin and flavonol aglycons, kaempferol and quercetin, and their glycosides (i.e. rutin).[1,4] Catechin is the flavonoid most abundant and, with the alkaloid boldine, is the main contributor to the antioxidant activity of

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boldo leaf extracts.[1,4] For this high catechin content of boldo leaves and its bioactivity, it has been suggested that quality control of boldo leaf has to combine the analysis of catechin as well as the characteristic aporphine alkaloids.[4]

In addition to its antioxidant properties, the boldine molecule has two major absorption peaks, at 280 and 302 nm.[2,5] The latter would confer boldine a UV light-filtering property relevant to a photo-protective action. Hidalgo *et al*.[6] showed that it displays a photo-protector effect against UV-B both *in vitro* and *in vivo* in mice. More recently, Rancan *et al*.[7] investigated the photo-filtering properties of boldine in humans. These authors observed that the topical application of boldine protected the skin against erythema formation. Also catechin and rutin, in our previous study, showed a protective effect on DNA damage induced by hydroxyl radicals (**.**OH) generated from UV photolysis of H_2O_2.[8] Exposure to ultraviolet radiation (UV-R) induces genotoxic effects that contribute not only to skin photoaging but also to skin carcinogenesis.[9] Ninety percent of skin cancer cases have been attributed to the solar UV radiation, particularly its UV-B component which is greatly absorbed by cellular DNA. Also, UV-B indirectly damages DNA through reactive oxygen species (ROS) formation, which facilitate the oxidation of DNA and premature skin ageing possibly resulting in skin cancer.[10] In view of these considerations, we analysed, using an HPLC method, a methanolic extract from leaves of *P. boldus* for boldine, catechin, quercetin and rutin content, and we examined its effect on pBR322 DNA cleavage induced by **.**OH generated from UV-photolysis of hydrogen peroxide (H_2O_2) and by nitric oxide (NO).

Boldine and flavonoids quercetin and catechin have been shown to exhibit anti-cancer activity in preclinical studies.[11–13] Our previous studies found that this methanolic extract exhibited comparable degrees of anti-growth effect on human cancer epithelial cell lines, probably by the induction of apoptosis.[14] Therefore, we also investigated the activity of this natural product against the human melanoma cell line, M14. Several biochemical parameters were tested, such as cell viability (3(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide, MTT, assay) and cell membrane integrity (lactate dehydrogenase, LDH, release).[15] For the detection of apoptosis, the evaluation of DNA fragmentation (COMET assay) and caspase-3 activity assay were employed.[15]

The molecular chaperone Hsp70 acts at multiple steps in a protein's life cycle, including the processes of folding, trafficking, remodelling and degradation. The protective presence of Hsp70 can be beneficial for the whole organism, if Hsp70 is expressed in normal cells; however, in cancer cells, Hsp70 is a negative prognostic marker.[16] In cancer cells, the expression of Hsp70 is abnormally high, and Hsp70 may participate in oncogenesis and in resistance to chemotherapy. Its tumorigenic potential seems to correlate with its anti-apoptotic ability.[17] However, only a small number of pharmacological Hsp70 inhibitors have been identified; these include the flavonol quercetin.[18] Therefore, in this study, a possible relationship between Hsp70 expression and cell death elicited by extract from leaves of *P. boldus* in M14 cells, was investigated. The possible induction of oxidative stress was also evaluated by performing a fluorescent analysis of intracellular ROS production.[15]

Taken together, our results demonstrate, for the first time, that a methanolic extract of the leaves of *P. boldus*, for the synergistic activity of its components (boldine and flavonoids, catechin, quercetin and rutin, evidenced by HPLC analysis), is able to exhibit a protective effect on NO and UV radiation-induced DNA cleavage and to inhibit the growth of melanoma cells. In addition, our data seem to indicate that the apoptosis evoked by this natural product in M14 cells, at least in part, appears to be induced by a reduction of Hsp70 expression, associated with an increase of ROS production. The results obtained in our experimental conditions have more value, considering that, in all the parameters examined, the methanolic extract from leaves of *Peumus boldus* exhibited no effect on normal human cells.

Materials and Methods

Chemicals

All reagents were of commercial quality and were used as received. pBR322 plasmid DNA, diethylenetriaminepentaacetic acid (DTPA), 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, USA).

Plant material

The leaves of *P. boldo* were collected at Quintay (Valparaiso) in January 2006. A voucher specimen (voucher specimen No. 12–07) was deposited in the Department of Chemistry, Universidad Santa Maria, Valparaiso, Chile. The leaves of *P. boldo* were exhaustively extracted with methanol and concentrated under vacuum to a residue (yield 165 g (14.34%)).

Boldine, catechin, quercetin and rutin concentration was evaluated in the methanolic extract from leaves of *P. boldus* by chromatography, using an HPLC Waters system equipped with detection by diode arrangements. These compounds were identified by comparing their retention times (Rt) and spectra obtained with those of standards. For boldine the following conditions were used: dilution in methanol (33.6 mg in 20 ml), isocratic elution with methyl alcohol and phosphate buffer 0.025 M, at 1 ml/min. Detection was done at the maximum of the boldine spectra, 310 nm, Rt 2.1 min. For catechin a dilution of 50 mg in 10 ml of solvent (methanol : water 80 : 20), a system coupled in a series of two monolithic columns with precolumn of 10 mm and elution with solvent mixture (methanol : water 80 : 20) were used. Detection was done at 280 nm, Rt 11.5 min. For quercetin and rutin the following conditions were used: dilution in methanol (26 mg in 20 ml), isocratic elution with acetonitrile and phosphate buffer 0.025 M, at 1 ml/min (acetonitrile : phosphate buffer 80 : 20); detection was done at 370 nm. The retention times were: quercetin = Rt 18 min; rutin = Rt 4 min.

Activity in cell-free systems

DNA cleavage induced by hydrogen peroxide UV photolysis

The experiments were performed, as previously reported,[15] in a volume of 20 µl containing 33 µM in bp of pBR322 plasmid

DNA in 5 mM phosphate saline buffer (pH 7.4), and the methanolic extract from *P. boldus* leaves at different concentrations. Immediately before irradiating the samples with UV light, H_2O_2 was added to a final concentration of 2.5 mM. Untreated pBR322 plasmid was included as a control in each run of gel electrophoresis, conducted at 1.5 V/cm for 15 h. Gel was stained in ethidium bromide (1 µg/ml; 30 min) and photographed on Polaroid-Type 667 positive land film. The intensity of each scDNA band was quantified by means of densitometry. Parallel experiments, were also carried out in the presence of boldine and quercetin.

Analysis of DNA single-strand breaks induced by Angeli's salt

The experiments were performed, as previously reported,[15] by incubating pBR322 plasmid DNA in 100 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM DTPA, 0.15 mM Angeli's salt (prepared in 0.01 N NaOH), an appropriate amount of HCl to neutralise the NaOH present in the solution of Angeli's salt, and the methanolic extract from *P. boldus* leaves, at different concentrations, at 37°C for 1 h (final volume 10 µl, final pH 7.5). Untreated pBR322 plasmid was included as a control in each run of gel electrophoresis, conducted at 1.5 V/cm for 15 h. Gel was stained in ethidium bromide (1 µg/ml; 30 min) and photographed on Polaroid-Type 667 positive land film. The intensity of each scDNA band was quantified by means of densitometry. Parallel experiments, were also carried out in the presence of boldine, catechin, quercetin and rutin.

Study in cell culture

Cell culture and treatments

M14 human melanoma cells were grown in RPMI containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin and 25 µg/ml fungizone. Normal human non-immortalised buccal fibroblast cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 25 µg/ml fungizone. The cells were plated at a constant density to obtain identical experimental conditions in the different tests, thus to achieve a high accuracy of the measurements.

In the MTT assay the cells were plated at 6×10^3 cells per well for human cancer cells, and at 2×10^4 cells per well for normal human non-immortalised buccal fibroblast cells, in a 96-well flat-bottomed 200 µl microplate. In other tests, the cells were plated at 8×10^5 cells (2 ml) for human cancer cells, and 2×10^6 cells (2 ml) for normal human non-immortalised buccal fibroblast cells, per 35 mm culture dish.

After 24 h incubation at 37°C under humidified 5% carbon dioxide to allow cell attachment, the cells were treated with different concentrations of methanolic extract from *P. boldus* leaves and incubated for 72 h under the same conditions. This time of treatment was chosen since no effect of methanolic extract from *P. boldus* leaves was observed before 72 h of treatment, at least for parameters examined by us. In the MTT assay the cells were also exposed to pure compounds boldine, catechin, quercetin and rutin. Stock solutions of the extract and pure compounds were prepared in ethanol and the final concentration of this solvent was kept constant at 0.25%. Control cells received ethanol alone.

MTT bioassay

MTT assay was performed as described previously.[15] The optical density of each well sample was measured with a microplate spectrophotometer reader (Digital and Analog Systems, Rome, Italy) at 550 nm.

Lactate dehydrogenase release

LDH release was spectrophotometrically measured in the culture medium and in the cellular lysates at 340 nm by analysing NADH reduction during the pyruvate-lactate transformation, as previously reported.[15] The percentage of LDH released was calculated as 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.[15] Software (Leica-QWIN) allowed us to assess the quantitative and qualitative extent of DNA damage by measuring: (a) tail length (TL), intensity (TI) and area (TA); (b) head length (HL), intensity (HI) and area (HA). Finally, the program using these parameters calculates the level of DNA damage as: (a) the percentage of the fragmented DNA (TDNA) and (b) tail moment (TMOM). The tail moment is defined as the product of the percentage of DNA in the tail of the comet and TD value, which is obtained by calculating the distance between the centre of mass of the comet head and the centre of mass of the tail. The percentage of DNA in the comet tail was calculated as the rate of the fluorescence intensity in the comet tail relative to the total fluorescence; 100 randomly selected cells were analysed per sample.

Activity of caspase-3

The activity of caspase-3 was determined by using the Caspase colorimetric assay Kit (Sigma RBI, St Louis, USA). This assay measures the cleavage of a specific colorimetric caspase substrate, acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA). pNA (p-nitroaniline) is released from the substrate upon cleavage by caspase. Free pNA produces a yellow colour that is monitored by a Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) at $\lambda = 405$ nm. The caspase-3 activity was measured in cell lysates according to the manufacture's protocol. The total protein content, used to reflect cell number and measured as previously described,[15] was evaluated for each sample, and the results are reported as OD_{405} nm/mg protein and compared with control.

Western blot analysis

The expression of heat shock protein 70 (Hsp70) was evaluated by Western blot analysis. Briefly, the untreated and treated M14 cells were washed twice with ice-cold phosphate-buffered saline (PBS) and collected with lysing buffer (10 mM Tris-HCl plus 10 mM KCl, 2 mM $MgCl_2$, 0.6 mM Phenylmethanesulfonyl fluoride and 1% SDS, pH 7.4). After cooling for 30 min at 0°C, cells were sonicated. Twenty micrograms of total

protein, present in the supernatant, were loaded on each lane and separated by 4–12% Novex Bis-Tris gel electrophoresis (NuPAGE; Invitrogen, Milan, Italy). Proteins were then transferred to nitrocellulose membranes (Invitrogen, Italy) in a wet system. The transfer of proteins was verified by staining the nitrocellulose membranes with Ponceau S and the Novex Bis-Tris gel with Brillant blue R. Membranes were blocked in Tris-buffered saline containing 0.01% Tween-20 (TBST) and 5% non-fat dry milk at 4°C overnight. Mouse monoclonal anti-Hsp70 (1 : 200 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal anti-α–tubulin (1 : 5000 dilution) (Sigma, Milan, Italy) antibodies were diluted in TBST and membranes incubated for 24 h at room temperature. Antibodies were detected with horseradish peroxidase-conjugated secondary antibody using the enhanced chemiluminescence detection Supersignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, USA). Bands were measured densitometrically by ImageJ software and their relative density calculated based on the density of the α-tubulin bands in each sample. Values were expressed as arbitrary densitometric units corresponding to signal intensity.

Reactive oxygen species assay

ROS determination was performed by using a fluorescent probe (DCFH-DA) as previously described.[15] The fluorescence (corresponding to the radical species-oxidised DCF) was monitored spectrofluorometrically using a Hitachi F-2000 spectrofluorimeter (Hitachi, Tokyo, Japan): excitation 488 nm, emission 525 nm. The total protein content, measured as previously described,[15] was evaluated for each sample, and the results are reported as fluorescence intensity/mg protein and compared with control.

Statistical analysis

Each value represents the mean ± SD of three experiments performed in quadruplicate. Results were analysed using one-way analysis of variance 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, USA). Differences were considered significant at $P < 0.05$.

Results

Analysis of extract

The methanolic extract was subjected to HPLC analysis to determine boldine, catechin, quercetin and rutin (Figures 1–3). The identification of these compounds was evaluated by comparing their retention times (Rt) and spectra obtained with those of standards. The concentrations obtained for these compounds were: boldine 1.05%, Rt. 2.1 min; catechin 1.44%, Rt. 11.5 min; quercetin 0.1%, Rt. 18 min; rutin 0.14%, Rt. 4.0 min.

Biological activity in cell-free systems

The methanolic extract from *P. boldus* leaves was tested for its potential DNA-protective activity on pBR322 DNA cleavage induced by hydroxyl radicals (**.**OH), generated from UV photolysis of H_2O_2, and by NO. DNA derived from pBR322

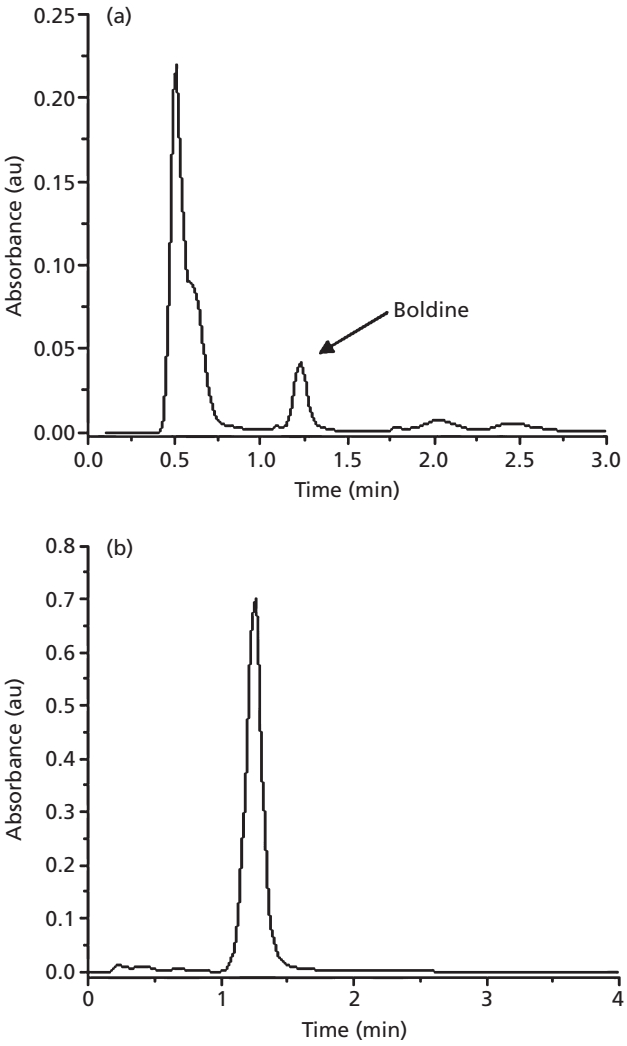

Figure 1 HPLC chromatograms at 310 nm of methanolic extract from *P. boldus* leaves (a) and standard solution of boldine (b).

plasmid showed two bands on agarose gel electrophoresis; the faster moving band corresponded to the native form of supercoiled circular DNA (scDNA) and the slower moving band was the open circular form (ocDNA). The UV irradiation of DNA in the presence of H_2O_2 suppresses the formation of scDNA, producing ocDNA and linear form (linDNA), indicating that **.**OH generated from UV photolysis of H_2O_2 produces DNA strand scission.[8,15] The addition of the extract, at 200–800 μg/ml concentrations, to the reaction mixture suppressed the formation of linDNA and induced a partial recovery of scDNA. In fact, the intensity of scDNA bands for plasmid DNA, treated with H_2O_2 in the presence of 800 μg/ml methanolic extract, was 94% (Table 1). The treatment of plasmid DNA with extract alone did not change the migration pattern (data not shown). To examine the role of the pure compounds, their effect was also investigated using the same experimental conditions. The results showed that boldine and quercetin, tested at a concentration (50 μM) greater than that present in the methanol extract from leaves of *P. boldus*, had no effect (Table 1). On the other hand, our previous study

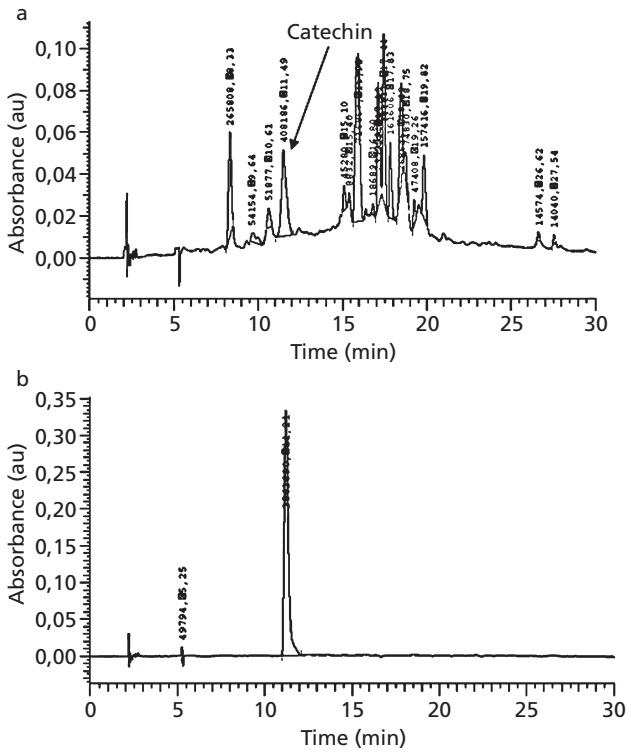

Figure 2 HPLC chromatograms at 280 nm of methanolic extract from *P. boldus* leaves (a) and standard solution of catechin (b).

reported that catechin and rutin exhibited a protective effect on plasmid DNA damage induced by UV photolysis of H_2O_2 only at doses higher than 200 μM.

Angeli's salt, a NO donor,[19] as previously reported,[15] induced in our experimental condition, a significant decrease in the scDNA band intensity (Table 2). The methanolic extract, at 200–800 μg/ml concentration, was able to reduce the NO-induced DNA damage, acting as a nitric oxide donor agent (Table 2). The treatment of plasmid DNA with extract alone did not change the migration pattern (data not shown). Also in this assay, pure compounds boldine, catechin, quercetin and rutin, tested at a concentration (50 μM) greater than that present in the methanol extract from leaves of *P. boldus*, exhibited no protective activity on NO-induced DNA damage (Table 2).

Study in cell culture

The methanolic extract from *P. boldus* leaves was tested *in vitro* for its potential activity against cell growth in human melanoma cells, M14, using MTT assay. The results, summarised in Figure 4, show that the methanolic extract at 5–40 μg/ml concentrations, exhibited a significant inhibitory effect ($P < 0.001$) on M14 cell growth. In particular, the vitality was 4% in cells exposed to 40 μg/ml concentration. Interestingly, the natural product examined, in our experimental conditions (5–40 μg/ml), revealed no cytotoxic effect against normal human buccal fibroblast cells (Figure 4), considered a useful model to evaluate the cytotoxic effects of carcinogens[20] and the tumour-specific cytotoxicity of potential antineoplastic agents.[21]

In an attempt to elucidate the role of boldine and flavonoids, catechin, quercetin and rutin, in the potential cancer cell antigrowth activity of the extract from leaves of *P. boldus*, we also evaluated the effect of these pure compounds. The results obtained revealed that all compounds examined, also at concentrations greater than those present in the extract, were unable to affect the vitality of cancer cells (Figure 5). Only catechin, at 25 and 50 μM concentrations, showed a significant inhibitory effect ($P < 0.001$) on M14 cells.

LDH is a soluble enzyme located in the cytosol, which is released into the surrounding culture medium upon cell damage and lysis. Measuring LDH in the culture medium can therefore be used as an indicator of membrane integrity, and thus a measurement of cytotoxicity.[15] No statistically significant increase in LDH release was observed in M14 cells treated with the methanolic extract from *P. boldus* leaves at 5 and 10 μg/ml concentrations (Table 3). Conversely, a significant increase in LDH was observed at higher concentrations (20 and 40 μg/ml) (Table 3).

Nuclear DNA was analysed using single-cell gel electrophoresis (SCGE), known as COMET assay, a sensitive method for the visualisation of DNA damage measured at the level of individual cells. The COMET assay also allows us to distinguish apoptotic from necrotic cells based on the DNA fragmentation pattern.[22] 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 condition, is reported as TDNA and TMOM in Table 4. The results clearly show an increase in both TDNA and TMOM at 5 and 10 μg/ml concentrations. These findings seem to suggest that the extract from *P. boldus* leaves induced, also at lower concentration of 5 μg/ml, cell death by apoptosis, because data in the literature indicate that only COMETS with high values of TMOM can be related to apoptosis.[23]

Active caspases cleave several important intracellular proteins, leading to the morphological and biochemical changes associated with apoptosis, such as oligonucleosomal fragmentation of chromosomal DNA.[24] Caspase-3 is the major executioner caspase in the caspase cascade, therefore experiments were performed to characterise the role of activation of this protein in cell growth inhibition mediated by tested extract. As shown in Figure 6, the activity of caspase-3, measured by pNA (p-nitroaniline), released from the specific caspase substrate, and reported as OD_{405} nm/mg protein, was significantly increased in M14 cells treated with the methanolic extract at 5 and 10 μg/ml concentrations, supporting the hypothesis that the cell growth inhibition, demonstrated in these experimental conditions, was correlated to an early signal of apoptosis. Conversely, at 20 and 40 μg/ml concentrations, the activity of this protease returned to control values.

We also tested the effect of this natural product on normal human non-immortalised fibroblast cells. At 5–10 μg/ml, the extract was ineffective in inducing apoptosis in these cells, as demonstrated by the results presented in Table 4 (COMET assay) and Figure 6 (caspase-3 activity). Also at 20 and 40 μg/ml concentrations, this natural product did not exhibit cytotoxic activity; in fact the LDH release was unmodified with respect to the values observed in control untreated cells (Table 3).

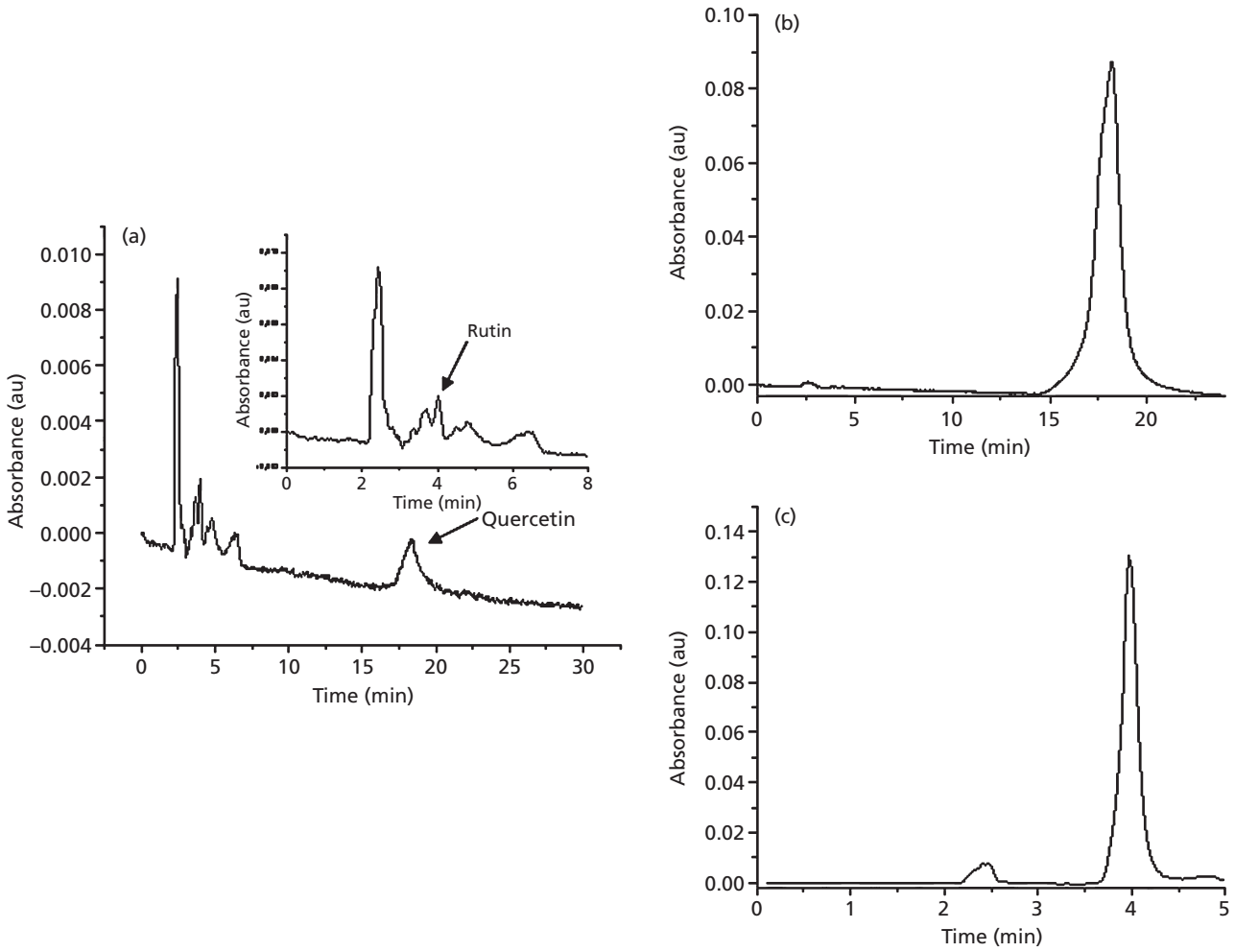

Figure 3 HPLC chromatograms at 370 nm of methanolic extract from *P. boldus* leaves (a) and standard solutions of quercetin (b) and rutin (c).

Table 1 Effect of methanolic extract from *P. boldus* leaves (BOE) and pure compounds, boldine and quercetin on DNA cleavage induced by the photolysis of H_2O_2

Treatment	Densitometric units of supercoiled DNA (% of native DNA)
scDNA	100
BOE	
200 μg/ml	44 ± 4.6*
400 μg/ml	83 ± 7.1
800 μg/ml	94 ± 6.3
Boldine	
50 μM	–
Quercetin	
50 μM	–

The hydroxyl radicals generated by the photolysis of H_2O_2 suppressed the supercoiled DNA (scDNA). The values are expressed as densitometric units obtained by scanning the agarose gel electrophoresis photos. Each value represents the mean ± SD of three experiments performed in quadruplicate. *$P < 0.001$ vs supercoiled DNA.

Hsp70, because of its chaperone function, has been shown to affect the apoptotic process and thereby increases the survival of cells exposed to a wide range of lethal stimuli. Hsp70 has also been shown to act as an inhibitor of apoptosis in cancer cells, increasing the tumorigenicity of these cells.[16,17] Therefore, a possible relationship between Hsp70 expression and cell death elicited by extract from leaves of *P. boldus* in M14 cells, was investigated. In Figure 7 the results of Hsp70 immunoblotting are reported. The data show a reduction of levels of this protein in M14 cells treated with the methanolic extract from *P. boldus* leaves at 5–20 μg/ml concentrations, compared with the values of control untreated cells. Hsp70 expression was undetectable in cancer cells exposed to extract at 40 μg/ml concentration. (data not shown). In normal human fibroblast cells, the level of Hsp70 expression was unmodified at all concentrations tested (5–20 μg/ml) (Figure 7).

ROS have been reported to be involved in cell death induced by a variety of stimuli and different antitumoral agents. We therefore examined whether tested extract-induced cell death may be correlated at an elevation of ROS. To assess changes in intracellular ROS levels, we employed an oxidation-sensitive fluorescent probe DCFH-DA. DCFH-DA can be taken up into cells, and then oxidised by ROS to its

Table 2 Effect of methanolic extract from *P. boldus* leaves (BOE) and pure compounds boldine, catechin, quercetin and rutin on Angeli's salt-mediated DNA damage

Treatment	Densitometric units of supercoiled DNA (% of native DNA)
scDNA	100
Angeli's salt	
0.2 mM	8.0 ± 0.7*
BOE	
200 µg/ml	37 ± 4.6*,**
400 µg/ml	56 ± 6.6*,**
800 µg/ml	83 ± 5.5**
Boldine	
50 µM	–
Catechin	
50 µM	–
Quercetin	
50 µM	–
Rutin	
50 µM	–

The values are expressed as densitometric units obtained by scanning the agarose gel electrophoresis photos. Each value represents the mean ± SD of three experiments performed in quadruplicate. *$P < 0.001$ vs supercoiled DNA; **$P < 0.001$ vs Angeli's salt-treated DNA.

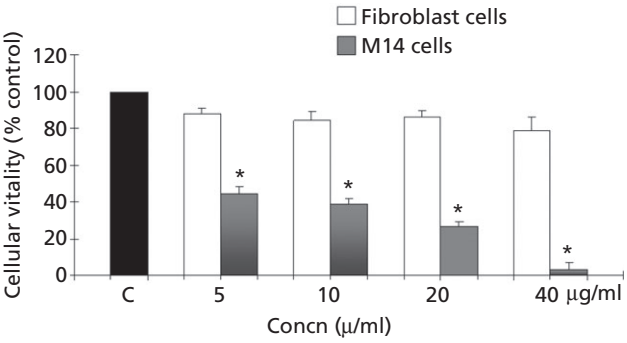

Figure 4 Cell growth, assayed using MTT test, of fibroblast and M14 cells untreated and treated with the methanolic extract from *P. boldus* leaves at different concentrations for 72 h. Stock solution of extract was prepared in ethanol and the final concentration of this solvent was kept constant at 0.25%. Control cultures received ethanol alone. Each value represents the mean ± SD of three experiments, performed in quadruplicate. *$P < 0.001$ vs control untreated cells.

fluorescent derivative DCF. We found that the DCF fluorescence increased in a concentration-dependent manner in M14 cells treated with the extract from *P. boldus* leaves (Figure 8). Alternatively, also in this assay the extract exhibited insignificant effect on fibroblast cells, also at higher concentration of 40 µg/ml (Figure 8).

Discussion

The skin is the largest organ of the human body and a primary target for an array of environmental insults. As ozone is becoming thinner, our skin becomes more in contact with a variety of harmful agents such as induction of oxidative stress and exposure to UV-R. UV-R has been identified as a cause of several hazardous cutaneous effects, including immune suppression, dermatitis, premature aging and skin cancer. UV-R can cause direct biological damage, or indirect damage via the production of ROS. These cause oxidative damage to DNA, proteins and lipids.[25] The epidermis contains antioxidant defences including the enzymes, superoxide dismutase, glutathione peroxidase and catalase, which remove ROS from the skin. Increased production of ROS following exposure to UV-R can deplete these antioxidant defences, leaving the skin vulnerable to attack from ROS.[25] In addition, it has become clear that perturbations or defects in the signaling cascade of NO and reactive nitrogen intermediates have been shown to be associated with common forms of skin diseases. It has been reported that NO liberated following UV-R irradiation plays a significant role in initiating erythema and inflammation.[26] NO can combine with UV-induced superoxide to form peroxynitrite which exists in equilibrium with peroxynitrous acid. These reactive nitrogen species are very toxic, and can cause DNA damage, nitrosylation of tyrosine residues in proteins, and initiate lipid peroxidation, all of which interfere with cellular function.[26] Various compounds in foods as well as in medicinal plants have been widely used for wound-healing, anti-aging, and disease treatments in the skin. Their possible use in the prevention of skin cancer, including melanoma, has been suggested. The biological activity of these natural compounds, has been correlated in part to their capacity to contrast the oxidative and nitrosative stress.[26] Our results suggest that also the methanolic extract from leaves of *P. boldus,* containing boldine 1.05%, catechin 1.44%, quercetin 0.1% and rutin 0.14%, which were detected using a HPLC Waters system (Figures 1–3), could act in this way in skin protection. In fact, it exhibited protection against DNA damage induced by.OH radicals, generated by UV-photolysis of H_2O_2 (Table 1), and like carboxy-PTIO (2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt), a nitric oxide scavenger,[27] it was able to reduce NO-induced DNA single-strand breakage (Table 2). Under the same experimental conditions, boldine, catechin, quercetin and rutin, also at concentrations greater than those present in the extract, as previously reported[8] and shown in Tables 1 and 2, exhibited no protective activity on plasmid DNA damage, indicating that the extract capacity to prevent DNA damage is most likely attributed to the synergistic effect of different constituents. In the present study, we also show that only the extract from leaves of *P. boldus* inhibited the growth of M14 human cancer cell line (Figure 4). In fact, boldine, catechin, quercetin and rutin also at the concentrations greater than those present in the extract, were unable to affect the vitality of cancer cells (Figure 5). Only catechin at 25 and 50 µM concentrations showed a significant inhibitory effect ($P < 0.001$). On the other hand, the recent studies of Gerhardt *et al.*[11] reported that boldine, after 72 h of treatment, decreased the cell number of different glioma cell lines at doses higher than 80 µM, and in cell-type specific manner.

Interestingly, the extract from leaves of *P. boldus* exhibited no effect on the viability of normal fibroblast cells (Figure 4). These findings encouraged us to explore the mechanisms that may be involved in the selective growth inhibitory activity of the extract on M14 cells.

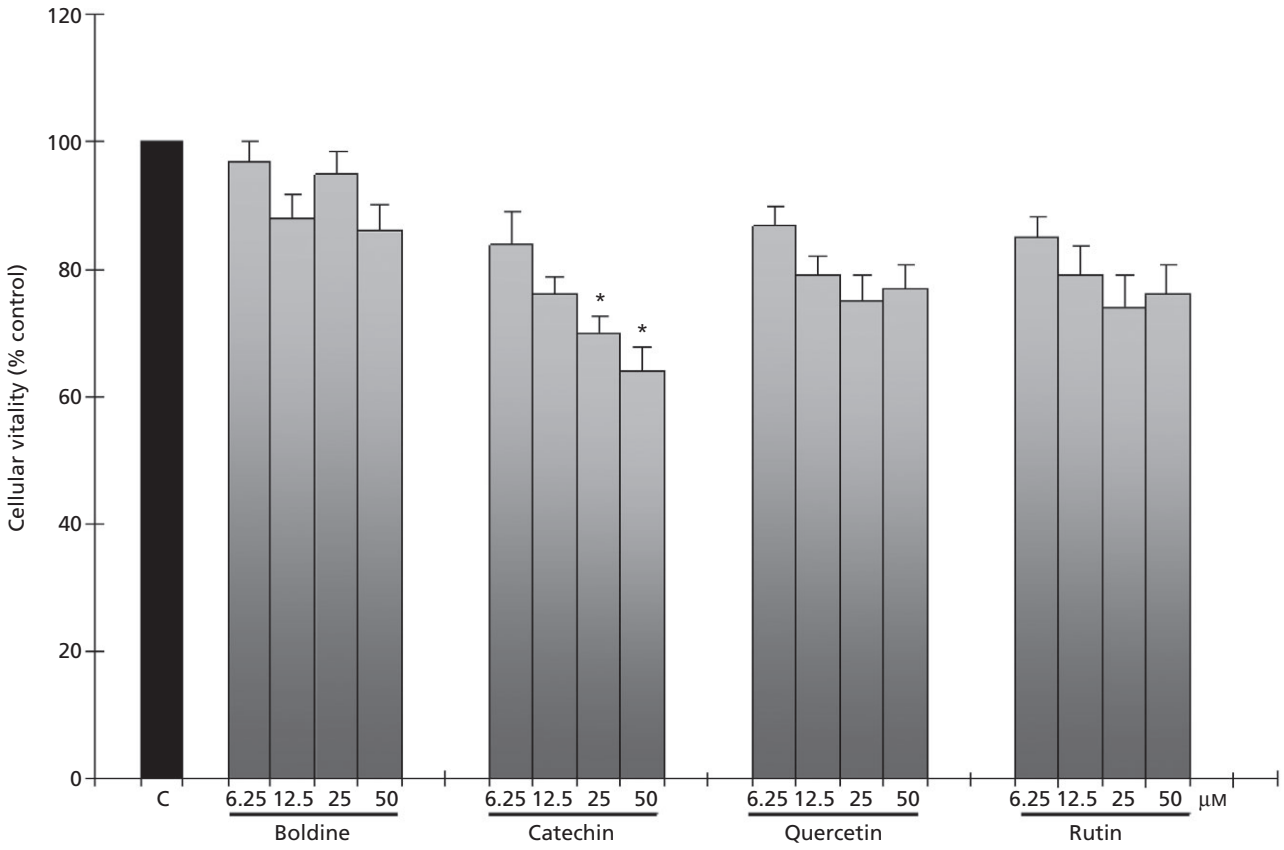

Figure 5 Cell growth, assayed using MTT test, of M14 cells untreated and treated with pure compounds boldine, catechin, quercetin and rutin at different concentrations for 72 h. Stock solution of pure compounds was prepared in ethanol and the final concentration of this solvent was kept constant at 0.25%. Control cultures received ethanol alone. Each value represents the mean ± SD of three experiments, performed in quadruplicate. *$P < 0.001$ vs control untreated cells.

Table 3 Lactate dehydrogenase (LDH) release, expressed as percentage of LDH released into the cell medium with respect to total LDH, in fibroblast and M14 cells treated with different concentrations of methanolic extract from *P. boldus* leaves (BOE)

Treatment	% LDH released
Fibroblast cells	
Control	5.0 ± 0.5
BOE	
5 μg/ml	7.2 ± 0.7
10 μg/ml	6.6 ± 0.5
20 μg/ml	8.3 ± 0.3
40 μg/ml	5.1 ± 0.4
M14 cells	
Control	8.0 ± 0.9
BOE	
5 μg/ml	9.7 ± 0.7
10 μg/ml	9.5 ± 1.2
20 μg/ml	40.1 ± 3.7*
40 μg/ml	86.3 ± 2.8*

The values are the mean ± SD of three experiments performed in quadruplicate. *$P < 0.001$ vs control untreated cells.

Table 4 COMET assay of genomic DNA of fibroblast and M14 cells untreated and treated with methanolic extract from *P. boldus* leaves (BOE)

Treatment	TDNA	TMOM
Fibroblast cells		
Control	26.5 ± 2.0	89 ± 4.2
BOE		
5 μg/ml	34 ± 3.0	75 ± 10
10 μg/ml	29 ± 6.0	96 ± 11
20 μg/ml	24 ± 2.1	82 ± 23
40 μg/ml	34 ± 2.7	98 ± 9
M14 cells		
Control	19.3 ± 3.0	91 ± 4.5
BOE		
5 μg/ml	105 ± 3.0*	1075 ± 15*
10 μg/ml	149 ± 6.0*	1135 ± 12*
20 μg/ml	49 ± 2.1*	323 ± 21*
40 μg/ml	45 ± 2.5*	327 ± 9*

TDNA, % of the fragmented DNA; TMOM, tail moment expressed as the product of TD (distance between head and tail) and TDNA. The values are the mean ± SD of three experiments performed in quadruplicate. *$P < 0.001$vs control untreated cells.

In cutaneous cells, there is a homeostatic relationship between cell proliferation and apoptosis. Alterations in either cell proliferation or cell death can lead to a loss of growth control, and thus play a major role in the process of tumorigenesis. Defects of apoptotic pathways influence also drug resistance, and because of these defects chemotherapy often fails. Recent studies have suggested that the resistance of human melanoma to apoptosis is an important mechanism underlying this cancer's aggressiveness and its poor response to chemotherapeutic agents. The induction of apoptosis in tumor cells is considered very useful in the management and therapy of cancer, including melanoma.[28] It is thus considered important to screen apoptotic inducers from plants, either in the form of extracts or as components isolated from them. Consistent with this approach, our data suggest that the methanolic extract from leaves of *P. boldus* is able to induce apoptosis in melanoma cancer cells. In fact, a high DNA fragmentation (Comet assay), occurred in M14 cells exposed to this extract at 5–10 μg/ml concentrations. One pathway of caspase activation is the intrinsic or mitochondrial pathway, from which cytochrome c is released into the cytosol, which interacts with cytosolic apoptosis protease-activating factor-1

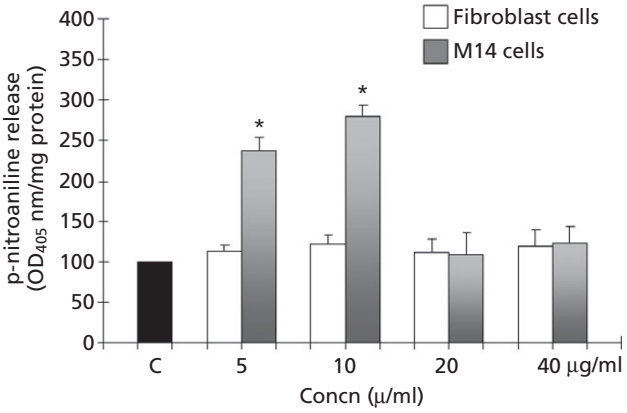

Figure 6 Caspase-3 activity, determined by using the Caspase colorimetric assay Kit (Sigma RBI St Louis, USA), in fibroblast and M14 cells untreated and treated with the methanolic extract from *P. boldus* leaves at different concentrations for 72 h. Stock solution of extract was prepared in ethanol and the final concentration of this solvent was kept constant at 0.25%. Control cultures received ethanol alone. Each value represents the mean ± SD of three experiments, performed in quadruplicate. $^{*}P < 0.001$ vs control untreated cells.

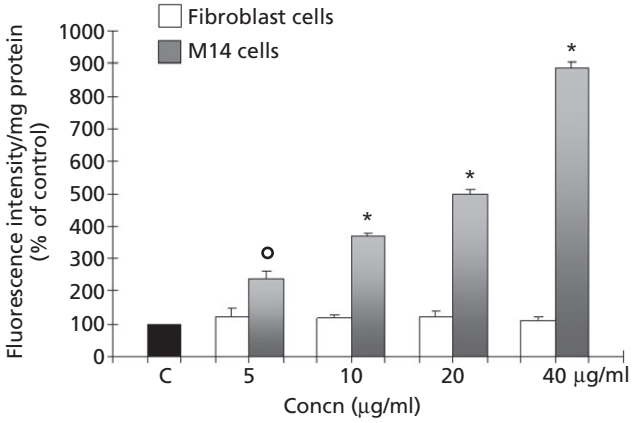

Figure 8 Reactive oxygen species (ROS) determination, performed by using a fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA), in fibroblast and M14 cells untreated and treated with the methanolic extract from *P. boldus* leaves at different concentrations for 72 h. Stock solution of extract was prepared in ethanol and the final concentration of this solvent was kept constant at 0.25%. Control cultures received ethanol alone. Each value represents the mean ± SD of three experiments, performed in quadruplicate. $^{\circ}P < 0.05$, $P < 0.001$ vs control untreated cells.

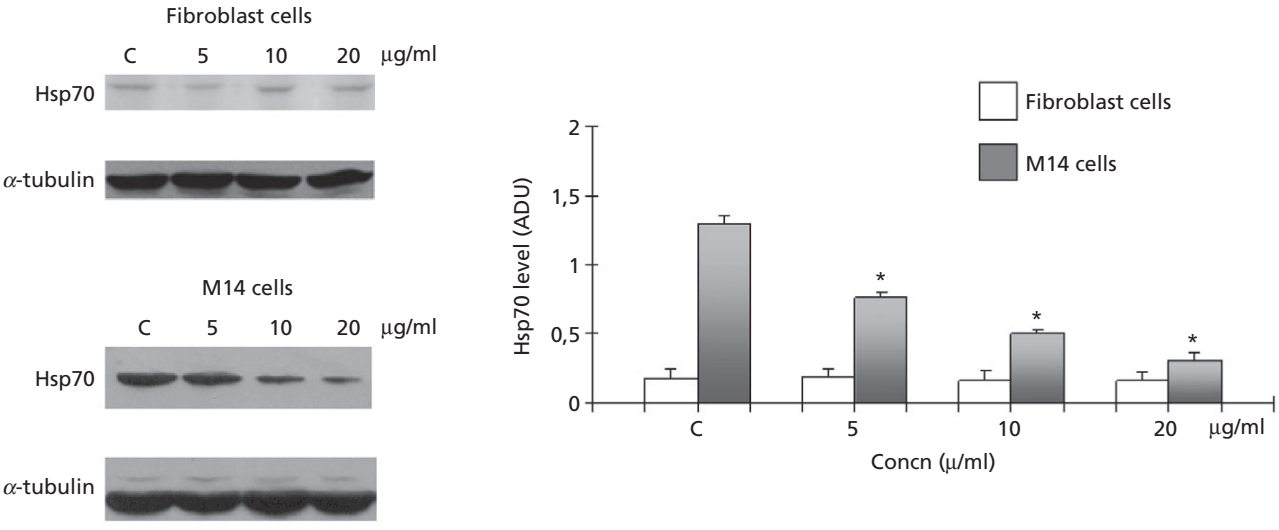

Figure 7 Levels of Hsp70 protein in fibroblast and M14 cells untreated and treated with the methanolic extract from *P. boldus* leaves at different concentrations for 72 h. Stock solution of extract was prepared in ethanol and the final concentration of this solvent was kept constant at 0.25%. Control cultures received ethanol alone. Values are expressed as arbitrary densitometric units (ADU) corresponding to signal intensity present on the autoradiography of Western blots. Each value represents the mean ± SD of three experiments, performed in quadruplicate. $^{*}P < 0.001$ vs control untreated cells.

(Apaf-1) and procaspase-9 to form the apoptosome, the caspase-3 activation complex.[17] Our results show that, following incubation with Boldo extract, caspase-3 activity was significantly ($P < 0.001$) increased in M14 cancer cell line (Figure 6), thus strongly suggesting that the mitochondrial apoptotic pathway is involved in Boldo extract-induced apoptosis. Alternatively, it was ineffective in inducing apoptosis in human non-immortalised fibroblast cells, as shown in Table 4 (COMET assay) and Figure 6 (caspase-3 activity).

The molecular chaperone Hsp70 acts at multiple steps in a protein's life cycle, including during the processes of folding, trafficking, remodeling and degradation. The protective presence of Hsp70 can be beneficial for the whole organism, if Hsp70 is expressed in normal cells, however in cancer cells, Hsp70 is negative prognostic marker.[16] In cancer cells, the expression of Hsp70 is abnormally high, and Hsp70 may participate in oncogenesis and in resistance to chemotherapy. Its tumorigenic potential seems to correlate with its ability to disable apoptosis. Antisense constructs of Hsp70 have been shown to sensitise cancer cells to apoptosis and to eradicate tumors in several models.[17] Elevated Hsp70 levels block the apoptotic pathway at different levels. Some studies have suggested that Hsp70 may inhibit apoptosis by acting downstream of mitochondria and cytochrome c release.[17] This anti-apoptotic effect was explained by the Hsp70-mediated modulation of the apoptosome.[17] Indeed, Hsp70 has been demonstrated to directly bind to the cytosolic apoptosis protease-activating factor-1 (Apaf-1), thereby preventing the recruitment of procaspase-9 to the apoptosome. Our data reinforce the well documented existence of a linkage between Hsp70 expression and cancer cell demise, and permit to hypothesise that the reduction of Hsp70 levels induced in M14 by methanolic extract from leaves of *P. boldus* could allow induction of apoptosis. In fact, the results obtained clearly demonstrate that *P. boldus* leaf extract, at 5 and 10 μg/ml, induced a reduction of Hsp70 expression (Figure 7) correlated with a high DNA fragmentation (Table 4) and a significant increase of the caspase-3 enzyme activity (Figure 6). Alternatively, at higher concentrations, the extract induced extreme damage, associated with a lower levels of Hsp70 expression (Figure 7), and evidenced by a different pattern of DNA damage (COMET assay) (Table 4), a high LDH release (Table 3) and a reduction in the caspase-3 activity (Figure 6). The release of cytochrome c from mitochondria and the inhibition of the mitochondrial respiratory chain was assumed to result in the overproduction of ROS, which would act as mediators of the death signaling pathway.[29] Studies have shown that the addition of ROS or the depletion of endogenous antioxidants can induce programmed cell death.[30] Our data suggest that the significant increase of ROS production, at 5–10 μg/ml concentrations, probably induced by Hsp70 down-modulation, could amplify the apoptosis cascades. Alternatively, at higher concentrations (20–40 μg/ml), when the capacity of the cells to sustain Hsp70 synthesis is reduced, our results seem to indicate that necrosis cell death, associated with a high LDH release (Table 3), and demonstrated by COMET assay values (Table 4), was induced by a further increase in ROS production, generating intolerable oxidative stress in cancer cells that are already near a threshold for tolerating ROS.[31] Our hypothesis is further confirmed by caspase-3 activity results, demonstrating a reduction in the activity of this protease at higher concentration, 20 and 40 μg/ml (Figure 6). Our data obtained with normal fibroblast cells also seem to support the existence of a correlation between the Hsp70 down-modulation and a modification of intracellular redox state. In fact, according to previous studies showing that the cytotoxic effect of Hsp70 down-modulation is particularly strong in transformed cells and undetectable in normal cells,[17] boldo extract treatment exhibited no activity on human normal fibroblast cells. This natural product had no effect on ROS production at 5 and 10 μg/ml concentrations, neither did it induce a stress response at higher concentrations (20 and 40 μg/ml), as demonstrated by LDH release evaluation (Table 3), COMET assay (Table 4) and Hsp70 levels (Figure 7). On the other hand, while it has been reported that phenolic phytochemicals have antioxidant/protective properties in normal tissues and cells, they can, paradoxically, induce the formation of ROS to achieve an intolerable level of high oxidative stress in some cancer cells.[31]

Conclusions

Our data provided the first evidence that boldo, known from ancient times for its health-beneficial characteristics, for the synergistic effect of different constituents boldine and flavonoids, is able to contrast UV light and NO-mediated DNA damage. Therefore, they suggest its possible use for the prevention of afflictions correlated to UV-R, such as skin cancer, including melanoma.

The extract from leaves of *P. boldus* has also shown interesting potential anti-tumour activity. Our results, in fact demonstrate the capacity of this natural product, but not of pure compounds boldine and flavonoids, catechin, quercetin and rutin, to selectively attenuate the growth of M14 cells. The central and novel finding in this pre-clinical study is that apoptosis induced by this natural product in M14 cells appears to be mediated, at least in part, via the inhibition of Hsp70 expression, which may be correlated with a modulation of redox-sensitive mechanisms. In addition, we have provided further support that Hsp70 confers resistance to apoptosis in melanoma cancer cells. Therefore, the combination of boldo with other anti-melanoma therapies could be considered a promising strategy that warrants further in-vivo evaluation.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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