

***Leccinum vulpinum* Watling induces DNA damage, decreases cell proliferation and induces apoptosis on the human MCF-7 breast cancer cell line**

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ABSTRACT

The current work aimed to study the antitumour activity of a phenolic extract of the edible mushroom *Leccinum vulpinum* Watling, rich essentially in hydroxybenzoic acids. In a first approach, the mushroom extract was tested against cancer cell growth by using four human tumour cell lines. Given the positive results obtained in these initial screening experiments and the evidence of some studies for an inverse relationship between mushroom consumption and breast cancer risk, a detailed study of the bioactivity of the extract was carried out on MCF-7 cells. Once the selected cell line to precede the work was the breast adenocarcinoma cell line, the human breast non-malignant cell line MCF-10A was used as control.

Overall, the extract decreased cellular proliferation and induced apoptosis. Furthermore, the results also suggest that the extract causes cellular DNA damage. Data obtained highlight the potential of mushrooms as a source of biologically active compounds, particularly with antitumour activity.

Keywords: Mushrooms, phenolic extract, bioactive compounds, antitumour potential, apoptosis, DNA damage.

1. Introduction

It is recognized worldwide that mushrooms are an excellent food both from a nutritional and bioactive point of view (Cheung, 2010; Roupas et al., 2012; Wasser, 2011). Indeed, according to the literature, in addition to their nutritional value, mushrooms have also been recognized for their therapeutic value (Öztürk et al., 2015).

Among these health benefits, the antitumor potential of different mushroom species and/or their components is well recognized by the scientific community (Ferreira et al., 2010). Actually, there is some evidence (from case-control studies in humans subjected to controlled diets) of an inverse relationship between the consumption of mushrooms and breast cancer risk (Hong et al., 2008; Roupas et al., 2012; Shin et al., 2010; Zhang et al., 2009). Moreover, there are some studies attesting the beneficial effects of some mushroom extracts in cancer patients. An *Agaricus blazei* extract has been reported to have beneficial effects in gynecological cancer patients undergoing chemotherapy, maintaining their immune activities and improving their quality of life (Ahn et al., 2004). Furthermore, the efficacy of PSK (Polysaccharide-K, obtained from *Trametes versicolor*) as an adjuvant immunotherapy agent after surgery has been described, in patients with colorectal cancer by increasing the patients survival and reducing the recurrence risk (Ito et al., 2004; Mitomi et al., 1992; Ohwada et al., 2004; Torisu et al., 1990), as well as in patients with gastric cancer (Nakazato et al., 1994; Tsujitani et al., 1992). In addition, dietary supplementation with an extract from *Agaricus sylvaticus* showed to be beneficial in postsurgical patients with colorectal cancer (Fortes et al., 2009).

The phenolic compounds are amongst the compounds with higher bioactivity (Öztürk et al., 2015). Indeed, the phenolic compounds, present in both wild and cultivated mushrooms, have been recognized as bioactive compounds which may be extracted for the purpose of being used as functional constituents (Cheung, 2010; Palacios et al.,

2011; Patel and Goyal, 2012; Reis et al., 2012; Vaz et al., 2012a). Likewise, mushrooms might be used directly in diet, promoting health benefits and taking advantage of the additive and synergistic effects of their bioactive compounds (Ferreira et al., 2009).

There are very few studies on the *Leccinum vulpinum* Watling mushroom species. To our knowledge, such studies have only outlined its morphological description (Ellis and Ellis, 1990), as well as some of its ecological (Iwański and Rudawska, 2007; Saxén and Ilus, 2008; Vaaramaa et al., 2009) and enzymatic characteristics (Chen et al., 2001 and 2003).

Taking into account the facts aforementioned regarding the potential of mushrooms and their components, and since there are no studies on *L. vulpinum* bioactivity, in the present work the antitumour potential of a phenolic extract obtained from this species was evaluated. The extract was initially chemically characterized by chromatographic techniques so as to measure their phenolic acids content. Afterwards, the extract was tested against a panel of four human tumour cell lines, in order to perform a first assessment of its effect on the growth of human tumour cell lines. Moreover, some functional assays were performed in order to further evaluate the effects of the phenolic extract on the MCF-7 cells (*i.e.*, cell proliferation, cell cycle profile and apoptosis). Additionally, the effects of the extract as an inducer of DNA damage were also evaluated, given that DNA damage is a mechanism of action of several cytotoxic drugs, which may lead to cell cycle arrest (inhibiting proliferation) and apoptosis.

2. Material and methods

2.1. Mushroom species and samples preparation

Leccinum vulpinum Watling wild samples were collected in Bragança (Northeast of Portugal) in the autumn of 2012. Authentications were undertaken at the Polytechnic

Institute of Bragança and voucher specimens were deposited at the herbarium of the School of Agriculture of the Polytechnic Institute of Bragança, Portugal.

All samples were lyophilised (FreeZone 4.5 model 7750031, Labconco, Kansas City, MO, USA), reduced to a fine dried powder (20 mesh), mixed and stored in a desiccator, protected from light.

2.2. Extraction procedure and chromatographic characterization of the extract

The extraction procedure and chromatographic characterization were performed according to the procedure described by other authors (Reis et al., 2012). Briefly, approximately 1.5 g of lyophilized sample was extracted with methanol:water (80:20, v/v; 30 mL) at -20°C. The analysis of phenolic compounds contained in the sample was performed by ultra fast liquid chromatography (UFLC, Shimadzu 20A series; UFLC, Shimadzu Cooperation, Kyoto, Japan) and the detection was carried out in a photodiode array detector (PDA), using 280 nm as the preferred wavelength. The phenolic compounds present in the studied extract were characterised according to their UV and retention times compared with commercial standards. The quantitative analysis of the compounds, was based on the calibration curve obtained by the injecting of known concentrations of each standard compound: cinnamic acid, gallic acid, *p*-hydroxybenzoic acid and protocatechuic acid. The results were expressed in µg per g of extract.

For the biological assays, the residue extracts were re-dissolved in distilled water (8 mg/mL).

2.3. Screening for the cell growth inhibitory activity in human tumour cell lines and cytotoxicity evaluation

The screening for tumour cell growth inhibitory activity was carried out using the sulforhodamine B (SRB) assay, a procedure adopted by the NCI's *in vitro* anticancer drug screening (Neves et al., 2011; Vichai and Kirtikara, 2006). This colorimetric assay estimates the cell number indirectly, by staining cellular protein with SRB. Four human tumour cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HCT-15 (colorectal adenocarcinoma) and AGS (gastric adenocarcinoma). The choice of the cell lines was performed in accordance with the mortality rate of the cancer type. As reported by the Cancer Research UK (data from 2012; Cancer Research UK, 2014), lung cancer is definitely the most common cause of cancer deaths, in both men and women. It accounts for just about 23% of all male deaths and 21% for female deaths. The second leading cause of cancer death in women, is the breast cancer (15% of all female cancer deaths), therefore the importance of its study. HCT-15 cell line was chosen since the bowel cancer is the 3th cause of death in both men and women, responsible for 10% of cancer deaths. Although stomach cancer mortality is strongly related to age, this type of cancer is the 3rd most common cause of cancer death worldwide (considering both genders) and the 4th most common cause of cancer death in Europe. Therefore, this cell line was also selected to be included in the studied panel of human tumour cells. Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 5% heat-inactivated FBS, in a humidified air incubator containing 5% CO₂. Cells were plated in 96-well plates at an appropriate density (5.0×10^3 cells/well for MCF-7 and NCI-H460, 1.0×10^4 cells/well for HCT-15 and 7.5×10^3 cells/well for AGS) and allowed to adhere for 24 h. Since different cells have different growth rates, the optimal cell density to be plated for each cell line was previously optimised, based on NCI's *in vitro* anticancer drug screening with some alterations, in order to prevent the cells from reaching confluence during the duration of the assay (which *per se* would cause cell cycle arrest and apoptosis) (Azevedo et al.,

2013; Vaz et al., 2010). Cells were then incubated for 48 h with five serial dilutions of the extract, ranging from 12.5 µg/mL to 400 µg/mL (Lima et al., 2014). Following 48 h of incubation with the extract (or immediately for the T₀ plate), plates were fixed by adding ice-cold 10% trichloroacetic acid (w/v, final concentration; Panreac, Barcelona, Spain) and stained with 0.4 % SRB (Sigma Aldrich, St. Louis, MO, USA) in 1% (v/v) acetic acid. Bound dye was solubilised by adding 10 mM Tris base (Sigma Aldrich, St. Louis, MO, USA) and the absorbance was measured at 510 nm in a microplate reader (BioTek® Synergy HT, Winooski, VT, USA) (Vaz et al., 2012a). The results were expressed as GI₅₀ values (the concentration that inhibited cell growth by 50%) as described by Monks et al. (1991). Doxorubicin (tested concentrations from 1.25 x 10⁻⁸ to 2 x 10⁻⁷ M) and etoposide (tested concentrations from 9 x 10⁻⁷ to 14 x 10⁻⁶ M) were used as positive controls. The H₂O (vehicle used to dissolve the extracts) concentrations used (5%) were also tested (control treatments). Another control was included, consisting in cells growing in complete culture medium only (blank treatment).

For the cytotoxicity evaluation, the human breast non-malignant cell line MCF-10A was used. The cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA). MCF-10A cells were cultured in Dulbecco's modified Eagle's medium (DMEM): F12, supplemented with 5% heat inactivated horse serum, 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 20 ng/ml epidermal growth factor (EGF), and 100 ng/ml cholera toxin (Sigma-Aldrich, St Louis, MO). The cells were routinely cultured in a humidified atmosphere with 5% CO₂ and at 37°C and used in experiments when reached 70% – 80% confluence. Cells were plated in 96-well plates at the same density as MCF-7 (5.0 × 10³ cells/well) and allowed to adhere for 24 h. Cells were treated for 48 h with the different diluted sample solutions and a similar procedure described previously for the SRB assay was followed. The results were also expressed as GI₅₀ values and doxorubicin was used as a positive control.

2.4. Cell biological assays used to assess the antitumour activity

2.4.1. Preparation of cells for subsequent assays

MCF-7 cells were plated at 1.0×10^5 cells/well in 6-well plates and incubated for 24 h to adhere to the plates. Cells were then treated with the phenolic extract of *L. vulpinum* at its GI₅₀ and GI₇₅ concentrations for the following assays: cell proliferation, cell cycle analysis, apoptosis and analysis expression of some proteins (*i.e.*, p53, p21, Bcl-2, Bcl-xL, procaspase 9, PUMA, Bax, PARP, *p*-H2AX) which are associated with cell cycle and apoptosis. Blank cells (treated with medium) or control cells (treated with H₂O volumes corresponding to the ones used in the treatments with the extract) were also included. The percentage of viable cells in relation to Blank cells was also confirmed, with the trypan blue exclusion assay.

For the DNA damage assay, cells were treated with 200 and 250 µg/mL of the phenolic extract for 24 h. Blank and control cells were also included and the percentage of viable cells was also confirmed.

2.4.2. Cell proliferation analysis

Cell proliferation was analysed with the BrdU assay, according to the protocol previously described by [Palmeira et al. \(2010\)](#). Briefly, cells were treated for 48 h (Blank, control or with the different concentrations of the extract). One hour before harvesting, cells were incubated with 10 µM BrdU (Sigma Aldrich, St.Louis, MO, USA) and were then harvested and fixed in 4% paraformaldehyde (PFA; Panreac, Barcelona, Spain) in PBS for 30 min at room temperature. After centrifugation, cell pellets were re-suspended in PBS and stored at 4°C. Cell cytopins were prepared and incubated in 2 M HCl for 20 min. Following incubation with mouse anti-BrdU antibody (diluted 1/10; Dako) for 1 h at room temperature, cells were incubated with anti-mouse-

Ig-FITC (diluted 1/100; Dako) for 30 min at room temperature. Slides were then prepared with Vectashield mounting medium (with DAPI). BrdU incorporation was observed in a DM2000 microscope (LEICA; Wetzlar, Germany) and a semi-quantitative evaluation was carried out by counting a minimum of 500 cells per slide.

2.4.3. Cell cycle distribution analysis

Following the 48 h treatments, cells were harvested and fixed with ice-cold 70% ethanol and stored at 4°C for at least 12 h, until further analysis. Cells were re-suspended in PBS containing RNase A (0.1 mg/mL) and propidium iodide (5 µg/mL). Cellular DNA content was analysed by flow cytometry (BD Accuri™ C6 Flow cytometer, USA) and the percentage of cells in the G1, S and G2/M phases of the cell cycle (as well as the percentage of cells in the sub-G1 peak) was determined using the FlowJo 7.6.5 software (Tree Star, Inc., Ashland, OR, USA) after cell debris and aggregates exclusion and plotting at least 15 000 events per sample ([Vasconcelos et al., 2000](#)).

2.4.4. Apoptosis analysis

Induced apoptosis was assayed by Flow cytometry using the Human Annexin V-FITC/PI apoptosis kit (Bender MedSystems, Vienna, Austria), according to the manufacturer's instructions, as previously described ([Queiroz et al., 2013](#)). Flow cytometry was carried out using the flow cytometer mentioned above and plotting at least 15 000 events per sample. Data were analysed using the BD Accuri C6 Software (version 1.0.264.21).

2.4.5. Protein expression analysis

For analysis of protein expression, following 48 h of treatments, cell pellets were lysed in Winman's buffer (1% NP-40, 0.1 M Tris-HCl pH 8.0, 0.15 M NaCl and 5 mM

EDTA) complemented with protease inhibitor cocktail (Roche). The total protein content was quantified using the DC™ Protein Assay kit (Biorad, Hercules, CA), according to manufacturer's instructions. Proteins (20 µg) were loaded on 12% SDS-PAGE gel and transferred into a nitrocellulose membrane (Amersham, Pittsburgh, PA, USA). The following primary antibodies were used: mouse anti-p53 (1:5000; Santa Cruz Biotechnology), mouse anti-p21 (1:250; Calbiochem), mouse anti-PUMA (1:500; Santa Cruz Biotechnology), mouse anti-Bax (1:500; Santa Cruz Biotechnology), rabbit anti-caspase 9 (1:1000; Cell Signalling Technology), mouse anti-BCL₂ (1:100; Dako), rabbit BCL_{XL} (1:200; Santa Cruz Biotechnology), rabbit anti-PARP (1:2000; Santa Cruz Biotechnology), rabbit anti-p-H2A.X (1:200, Santa Cruz Biotechnology) and goat anti-actin (1:2000, Santa Cruz Biotechnology). The corresponding secondary antibodies were: anti-mouse IgG-HRP (1:2000, Santa Cruz Biotechnology), anti-rabbit IgG-HRP (1:2000, Santa Cruz Biotechnology) or anti-goat IgG-HRP (1:2000, Santa Cruz Biotechnology). The Amersham™ ECL Western Blotting Detection Reagents, the Amersham Hyperfilm ECL and the Kodak GBX developer and fixer were used for signal detection, as previously described ([Lima et al., 2006](#)).

2.4.6. Analysis of DNA Damage

DNA damage was analysed with the Alkaline Comet Assay 24 h after treatment with the phenolic extract or control treatments. This incubation period was selected (instead of 48 h) to evaluate the first DNA degradation stages that occurred, prior to cellular apoptosis. Hydrogen peroxide was used as positive control (1 mM) and it was added 10 min before cells harvesting. Harvested cells were stored in 10% DMSO in FBS at -80°C, following a procedure previously described by other authors ([Seca et al., 2014](#)). Briefly, after thawing, cells were washed with PBS, resuspended in 0.6% low melting point agarose and quickly poured onto slides, precoated with 1% agarose in water, with

the aid of a coverslip. Slides were kept 10 min on ice. After removing the coverslip, cells were lysed with ice-cold lysis buffer (100mM Na₂EDTA, 2.5M NaCl, 10mM Tris-HCl, pH 10.0, 1% Triton X-100) for 2 h, in the dark, at 4 °C, and then washed twice in ice-cold distilled water for 10 min. Slides were placed on an electrophoresis tank filled with electrophoresis buffer (10 M NaOH; 200mM Na₂EDTA) and incubated for 20 min to let DNA to unwind. Electrophoresis was carried out for 20 min at 23 V, 300 mA. After this period, slides were flooded with neutralisation buffer (0.4 M Tris, pH 7.5) for 20 min and then rinsed in ice-cold water for 10 min before being allowed to dry at room temperature overnight. Slides were rehydrated with distilled water (for 30 min), covered with 2.5 µg/mL of propidium iodide solution and incubated for 20 min at room temperature in the dark. Propidium iodide was rinsed with distilled water for 30 min and slides dried at 37°C. Comets were visualized using the fluorescent microscope mentioned above and representative photographs were taken. The analysis was made using the ‘Comet Assay IV v4.3’ imaging system (Perceptive Instruments). A minimum of 100 cells per condition were analysed.

2.5. Statistical analysis

All experimental data is presented as mean ± standard deviation (SD) or standard error (SE), from at least three independent experiments (most of them performed in duplicate). Statistical analysis was carried out using an unpaired student’s t-test. All analyses were performed comparing cells treated with the phenolic extract with Blank cells (cells incubated with medium only). *Indicates $p < 0.05$.

3. Results and discussion

3.1. Chromatographic characterization of the phenolic extract from L. vulpinum

To our knowledge, there are no studies concerning the chemical characterization of *L. vulpinum* extracts. Based on the statement that mushrooms and their components have numerous positive health benefits, in the present work it was intend to discover new bioactive extracts from unstudied species. For that, we tested a phenolic extract from *L. vulpinum* for its antitumour properties, initiating the study with its chemical characterization.

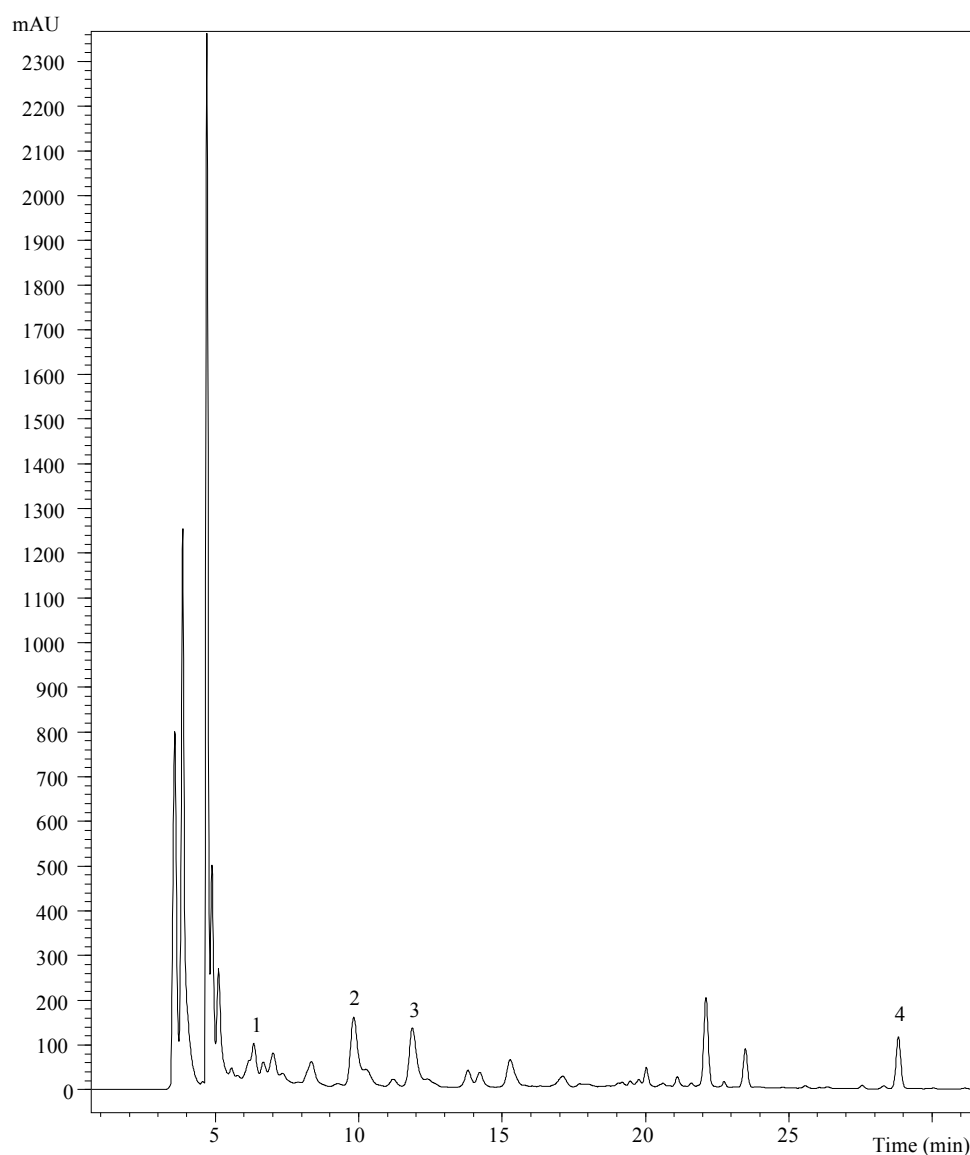
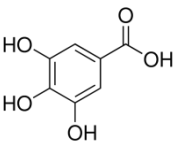
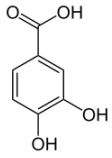
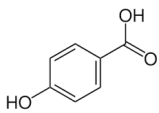
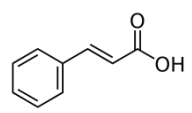


Fig. 1. Phenolic acids individual profile of the studied phenolic extract from *Leccinum vulpinum* Watling. 1- gallic acid; 2- protocatechuic acid; 3- *p*-hydroxybenzoic acid; 4- cinnamic acid.

Table 1

Phenolic acids and cinnamic acid present in the phenolic extract from *Leccinum vulpinum* Watling ($\mu\text{g/g}$ extract)

Gallic acid	Protocatechuic acid	<i>p</i> -Hydroxybenzoic acid	Cinnamic acid	Total phenolic acids
				
18.4 ± 0.3	77 ± 1	20 ± 1	38.0 ± 0.2	115.0 ± 1

Values correspond to the mean \pm SD.

As previously referred, mushroom phenolic compounds have been reported to contribute to the bioactivity of several species. The studied phenolic extract exhibited the presence of gallic acid ($18.4 \mu\text{g/g}$ extract), protocatechuic acid ($77 \mu\text{g/g}$ extract) and *p*-hydroxybenzoic acid ($20 \mu\text{g/g}$ extract; [Fig. 1](#); [Table 1](#)).

Accordingly, the main molecular group of phenolic compounds found in this mushroom specie was the hydroxybenzoic acids one, which comprises, besides *p*-hydroxybenzoic acid, gallic and protocatechuic acids. Gallic acid has been referred as having anticancer properties in several models such as prostate carcinoma DU145 and 22Rv1 cells ([Kaur et al., 2009](#)), non-small-cell lung cancer NCI-H460 cells ([Ji et al., 2009](#)), HeLa cervical cancer cells ([You et al., 2010](#)), leukaemia K562 cells ([Reddy et al., 2012](#)), and glioblastoma U87 and U251n cells, and having been pointed out as a valuable candidate for treatment of brain tumour ([Lu et al., 2010](#)). Protocatechuic acid has also been referred as having antitumour potential, namely as an inducer of cell death in HepG2 hepatocellular carcinoma cells ([Yip et al., 2006](#)), human breast cancer MCF-7 cells, lung cancer A549 cells, cervix HeLa cells, and prostate cancer LNCaP cells ([Yin et al., 2009](#)). Even though there are not many publications regarding the antitumour potential of *p*-hydroxybenzoic acid, it has been reported as a component of some phenolic

extracts with cytotoxicity/antitumour activity. This is the case of fermentation products of the medicinal plant *Houttuynia cordata* (Senawong et al., 2014) or honey samples (Spilioti et al., 2014). In some studies with mushrooms, the synergistic effects concerning the cytotoxicity of phenolic acids in human tumour cell lines have been reported, even when the isolated compounds (e.g. *p*-hydroxybenzoic acid) do not have such activity on their own (Heleno et al., 2014; Vaz et al., 2012a). Thus, *p*-hydroxybenzoic acid may exert antitumour effects when associated with other compounds, since there is also evidence that their own derivatives have antiproliferative and proapoptotic activities (Seidel et al., 2014).

Cinnamic acid was also found in the studied mushroom extract (38.0 µg/g extract). Cinnamic acid has been also reported as having anticancer properties. This natural compound inhibits the proliferation and modulates brush border membrane enzyme activities in colorectal adenocarcinoma Caco-2 cell line (Ekmekcioglu et al., 1998).

Considering these aspects, the presence of the mentioned phenolic compounds (and of the related compound cinnamic acid) in *L. vulpinum* phenolic extract is possibly responsible for its bioactive properties.

3.2. Cell growth inhibitory activity of the phenolic extract from L. vulpinum in human tumour cell lines and cytotoxicity in MCF-10A cells

There are some reports on tumour cell growth inhibitory activity of some phenolic extracts/compounds from other species of mushrooms (Heleno et al., 2014; Patel and Goyal, 2012; Vaz et al., 2012a). Nevertheless, to our knowledge such activity has never been previously investigated in the phenolic extracts of the *L. vulpinum* species. Therefore, in this work its effect on tumour cell growth inhibition was evaluated using a panel of four human tumour cell lines (MCF-7, NCI-H460, HCT-15 and AGS).

Table 2

Determined GI₅₀ concentrations of the phenolic extract from *Leccinum vulpinum* Watling

	HCT-15	NCI-H460	AGS	MCF-7	MCF-10A
GI ₅₀ values	53 ± 6 µg/mL	116 ± 8 µg/mL	135 ± 2 µg/mL	99 ± 11 µg/mL	297 ± 9 µg/mL
	Positive controls				
	Etoposide 6.8 ± 0.6 µM	Doxorubicin 25 ± 2 nM	Doxorubicin 23 ± 2 nM	Doxorubicin 26 ± 3 nM	Doxorubicin 19 ± 3 nM

Values correspond to the mean ± SE of three independent experiments, carried out with duplicates.

The extract inhibited the cell growth of all the cell lines tested (Table 2) and the lowest GI₅₀ concentrations were obtained in HCT-15 and MCF-7 cell lines. Positive controls were used for all the tested cells. Doxorubicin and etoposide are both used in cancer chemotherapy (Varaprasad et al., 2010; Chabner and Longo, 2011). Doxorubicin was selected as the main positive control; however, since the HCT-15 cell line was not very sensitive to this drug, presenting GI₅₀ values higher than the maximum tested (200 nM), etoposide was used as a positive control in this case.

It was decided to continue the studies in the MCF-7 cells since, as aforementioned, there is evidence for an inverse relationship between mushroom consumption and breast cancer risk.

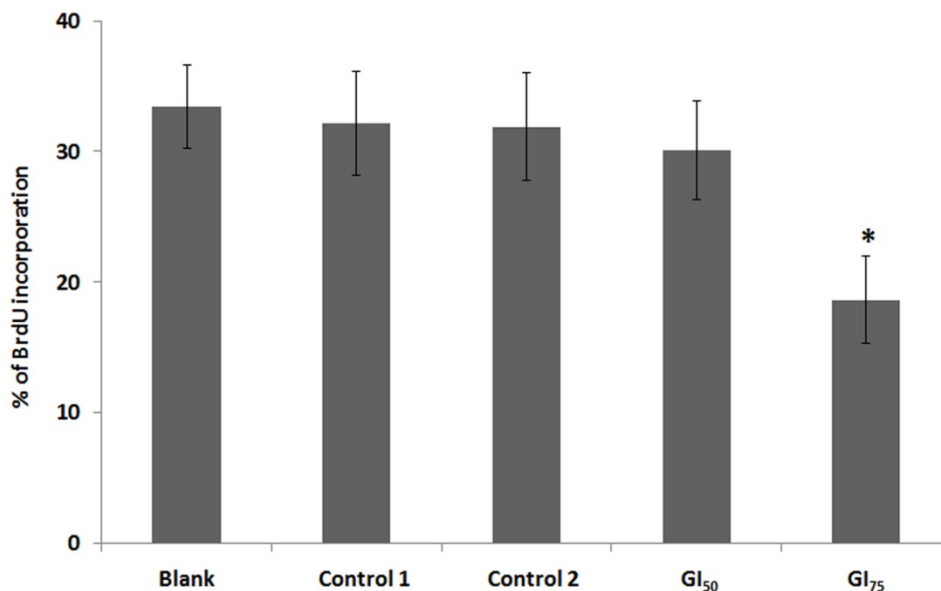
In order to assess the safety of using this phenolic extract, its cytotoxicity was tested in the human breast non-malignant cell line, MCF-10A. Although the phenolic extract of *L. vulpinum* exhibited some cytotoxicity on these cells, the values of GI₅₀ obtained were

much higher than those obtained in the breast tumour (MCF-7) cells (three times higher; [Table 2](#)). Therefore, the phenolic extract from *L. vulpinum* inhibited the growth of different human tumour cell lines, but was less toxic to the non-tumourigenic breast cancer cell line.

3.3. Effects of the phenolic extract of *L. vulpinum* on the human MCF-7 breast cancer cell line

L. vulpinum phenolic extract decreased proliferation of MCF-7 cells

There are some reports regarding the effect of mushroom extracts of other mushroom species on the proliferation of human tumour cell lines ([dos Santos et al., 2013 and 2014](#); [Yu et al., 2014](#)). In order to verify if the *L. vulpinum* phenolic extract interfered with the proliferation of MCF-7 cells, the levels of BrdU incorporation were analysed following 48 h treatment. As shown in [Fig. 2](#), the studied extract reduced cellular proliferation. Nevertheless, the effect on cell proliferation was only considered statistically significant with the GI₇₅ concentration (159 µg/mL). The fact the GI₅₀ concentration of the extract did not cause a statistically significant reduction on cell proliferation, may be due to an effect on cellular apoptosis at that concentration (see section 3.3.3. below) rather than an effect on cell cycle (proliferation). Indeed, it is possible that, although there is a decrease in the cell number following treatment with the



GI₅₀ concentration, the % of cells in the S-phase (which incorporate BrdU) is not altered.

Fig. 2. Cellular proliferation of MCF-7 cells treated with a phenolic extract of *Leccinum vulpinum* Watling analysed by the BrdU incorporation assay. Cells were treated with the GI₅₀ and GI₇₅ concentrations of the phenolic extract for 48 h. Appropriate controls were included: untreated cells (Blank) and cells treated with the corresponding volumes of the extract solvent (H₂O; Control 1 and Control 2). Results represent the mean ± SE of three independent experiments. *Represents $p < 0.05$ when comparing the effect of the studied phenolic extract with the Blank cells.

L. vulpinum phenolic extract did not alter cell cycle distribution of MCF-7 cells

Once differences in cellular proliferation were observed, the next step was to determine whether the extract caused cell cycle arrest. As previously referred, MCF-7 cells were treated with the GI₅₀ and GI₇₅ concentrations of the phenolic extract under study. From the analysis of the results obtained, no significant differences were observed in the different phases of the cell cycle (Fig. 3).

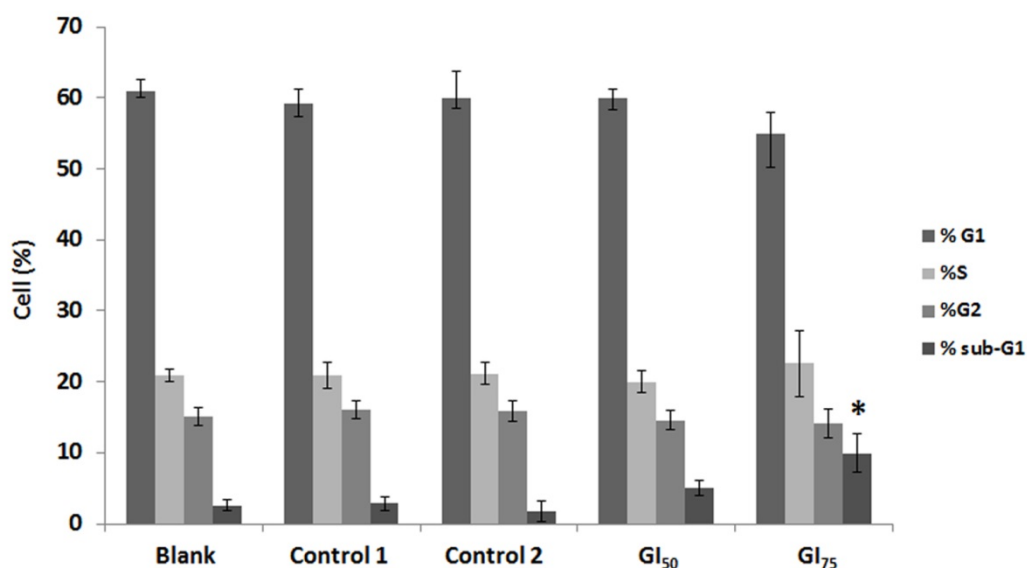


Fig. 3. Cell cycle analysis of MCF-7 cells treated with a phenolic extract of *Leccinum vulpinum* Watling. Cells were treated with the GI₅₀ and GI₇₅ concentrations of the phenolic extract for 48 h. Appropriate controls were included: untreated cells (Blank) and cells treated with the corresponding volumes of the extract solvent (H₂O; Control 1 and Control 2). Results represent the mean \pm SE of at least three independent experiments. *Represents $p < 0.05$ when comparing the effect of the studied phenolic extract with the Blank cells.

Having decreased cellular proliferation, we would expect that the extract would cause cell cycle arrest. These discrepancies may also be justified by the fact that cells may be dying by apoptosis (see section 3.3.3. below). Indeed, even though no significant differences in the cell cycle profile were observed, there was a significant increase in the sub-G1 peak of the cells that had been treated with the GI₇₅ concentration of the extract, which is suggestive of cellular apoptosis. Nevertheless, this sub-G1 peak cannot be used as a conclusive marker of apoptosis (Kajstura et al., 2007). In conclusion, these results suggest that one of the possible mechanisms of action of this phenolic extract is the induction of apoptosis.

L. vulpinum phenolic extract induced apoptosis in MCF-7 cells

Due to the above mentioned results (sections 3.3.1 and 3.3.2), the apoptotic levels of MCF-7 cells treated with the phenolic extract of *L. vulpinum* were studied, with the Annexin V-FITC/PI flow cytometry assay. The results obtained are presented in [Table 3](#).

The studied phenolic extract increased apoptosis in MCF-7 cells that had been treated for 48 h. In Blank and control cells the apoptosis levels were approximately 6 to 9%, which were within the normal values usually obtained for this cell line ([Vaz et al., 2012b](#)). When cells were treated with the GI₅₀ concentration of the extract the percentage of cells undergoing apoptosis increased to 13.4% and when cells were treated with the GI₇₅ concentration of the extract the levels of apoptosis increased to 27%; in both cases, the increase was considered statistically significant ([Table 3](#)).

Although flow cytometry is a very sensitive method, in order to corroborate the results obtained, a fluorescence microscopic examination of cells was performed. Cellular treatment with the studied phenolic extract caused formation of apoptotic bodies (marked with arrows), which is more visible with the GI₇₅ treatment ([Fig. 4](#)).

Table 3

Percentage of MCF-7 cells undergoing programmed cell death following treatment with a phenolic extract of *Leccinum vulpinum* Watling

Treatment				
Blank	Control 1	Control 2	GI ₅₀	GI ₇₅
6 ± 1	8.3 ± 0.6	9 ± 1	13.4 ± 0.8*	27 ± 4*

Values correspond to the mean ± SE of three independent experiments. *Represents $p < 0.05$ when comparing the effect of the studied phenolic extract with the Blank cells.

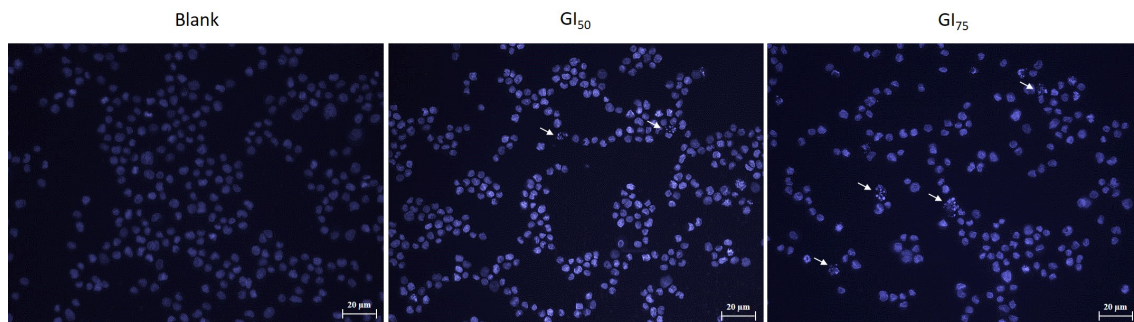


Fig. 4. Analysis of the apoptotic bodies in MCF-7 cells treated with a phenolic extract of *Leccinum vulpinum* Watling. Fluorescence microscopy images are representative of 3 independent experiments. Cell nuclei are stained with DAPI (blue). Arrows indicate apoptotic bodies; bar corresponds to 20 µm.

Therefore, it was confirmed that cellular treatment with this extract induced apoptosis.

In addition, the effect of the mushroom extract on the expression of some proteins involved in apoptosis was determined by Western blot (Fig. 5). Results show that treatment of MCF-7 cells for 48 h with the GI₇₅ concentration of the extract caused a significant increase in the p53 tumour suppressor protein. This protein becomes activated in response to stress conditions and induces p21 expression, which acts as a regulator of the cell cycle at the G1 checkpoint (Tsujitani et al., 2012). Accordingly, p21 levels were also found increased following cellular treatment with the GI₇₅ concentration of the extract.

Considering the verified increase in p53 levels and since p53 is a transcriptional factor which enhances the expression of genes involved in apoptosis such as Bax and PUMA, the cellular levels of Bax and PUMA were also evaluated (Charvet et al., 2011). No alterations were observed in Bax and PUMA expression levels with either of the treatments (GI₅₀ and GI₇₅; Fig. 5A and 5B). Being regulated by p53, their expression levels would be expected to be enhanced; however, the results obtained may be justified by the fact that only the 48h time point was analysed or that other mechanisms of apoptosis induction are being induced. Therefore, the levels of Bcl-2 and Bcl-xL were also evaluated by Western Blot. The cellular levels of both protein levels were reduced

following treatment with the extract (Fig. 5A), and in the case of Bcl-xL this reduction was found to be statistically significant for both concentrations of the extract (GI₅₀ and GI₇₅; Fig. 5B). The verified decrease in the Bcl-2 levels may be a cause or a consequence of the verified apoptosis. Indeed, apoptosis results in the activation of caspases which cause a series of cascade reactions that cleave essential proteins throughout the cell. Bcl-2 and Bcl-xL (anti-apoptotic proteins) may be cleaved by caspases, causing a reduction in their cellular levels and therefore further potentiating the apoptotic process (Fujita et al., 1998; Kirsch et al., 1999; Liang et al., 2002). So, the verified decrease in Bcl-2 and Bcl-xL may be a cause and/or a consequence of the verified increase in apoptosis.

Caspase-9 is the initiator caspase, whose activation is required for subsequent downstream caspase activation (Wu, 2014). Treatment of MCF-7 cells with the mushroom extract caused a decrease in procaspase-9 levels, suggesting that caspase-9 was activated. Usually, this decrease is accompanied by an appearance of a 37 kD cleavage product in the Western Blots (Sharma et al., 2012); however, we were not able to detect this band with the used antibody. Additionally, treatment with the GI₇₅ concentration of the phenolic extract significantly decreased total PARP levels (Fig. 5A and 5B), which is also typical of the activation of the mitochondrial pathway of apoptosis (Huang et al., 2012).

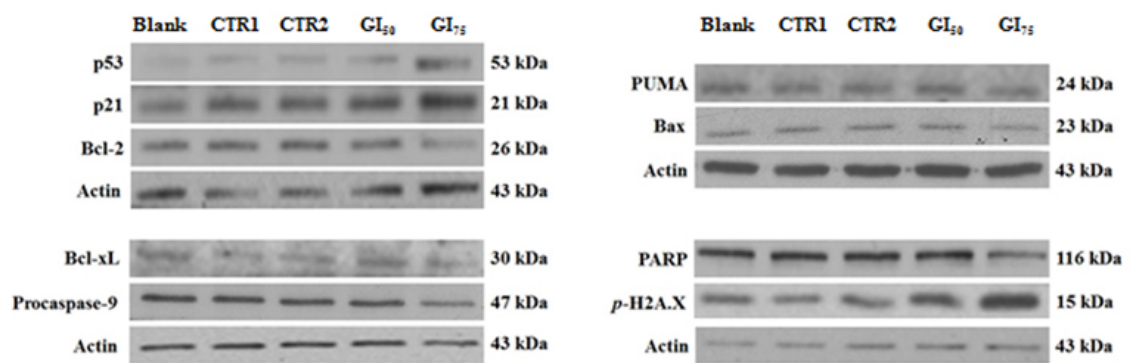
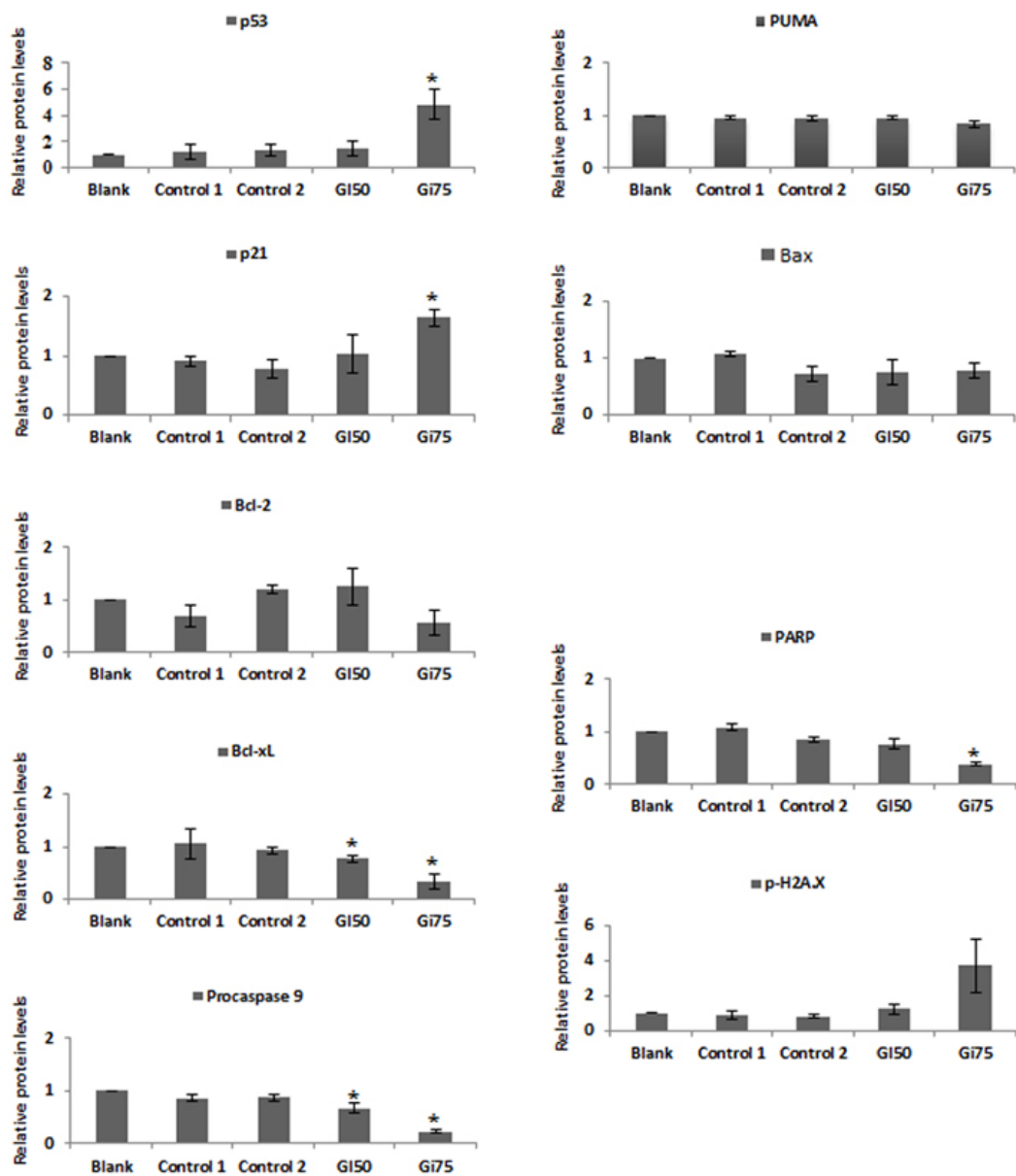
A**B**

Fig. 5. Expression of apoptosis- and DNA damage-related proteins in MCF-7 cells treated with a phenolic extract of *Leccinum vulpinum* Watling. (A) Western blot images representative of at least three independent experiments. Actin was used as a loading control. (B) Densitometry analysis of the Western blots. Cells were treated with the GI₅₀ and GI₇₅ concentrations of the phenolic extract for 48 h. Appropriate controls were included: untreated cells (Blank) and cells treated with the corresponding volumes of the extract solvent (H₂O; Control 1 (CTR1) and Control 2 (CTR2)). Results represent the mean \pm SE of three independent experiments and are expressed after normalization of the values obtained for each protein with the values obtained for actin and further expressed in relation to Blank cells. *Represents $p < 0.05$ when comparing the effect of the studied phenolic extract with the Blank cells.

L. vulpinum phenolic extract induced DNA damage

One of the causes of apoptotic cell death, namely via the intrinsic pathway, is DNA damage (Roos and Kaina, 2013). Accordingly, it was decided to analyze the levels of *p*-H2A.X, a protein also related to DNA damage and repair. Although the results observed in the Fig. 5A (Western blot) seem to indicate an increase in *p*-H2A.X levels, we found that this increase was not statistically significant (Fig. 5B).

In order to confirm if the mushroom extract induced DNA damage, the comet assay was performed. As aforementioned, cells were treated for 24 h with two different and high extract concentrations, 200 and 250 $\mu\text{g/mL}$ (both higher than the GI₇₅), in order to see the induction of DNA damage before the reduction in the number of viable cells due to apoptosis occurred (which would mean that a very small number of cells would be analysed). By measuring the percentage of tail DNA obtained, a significant increase upon treatment with 250 $\mu\text{g/mL}$ of the extract was observed (Fig. 6). Therefore, it can be concluded that the phenolic extract from *L. vulpinum* causes effectively DNA damage.

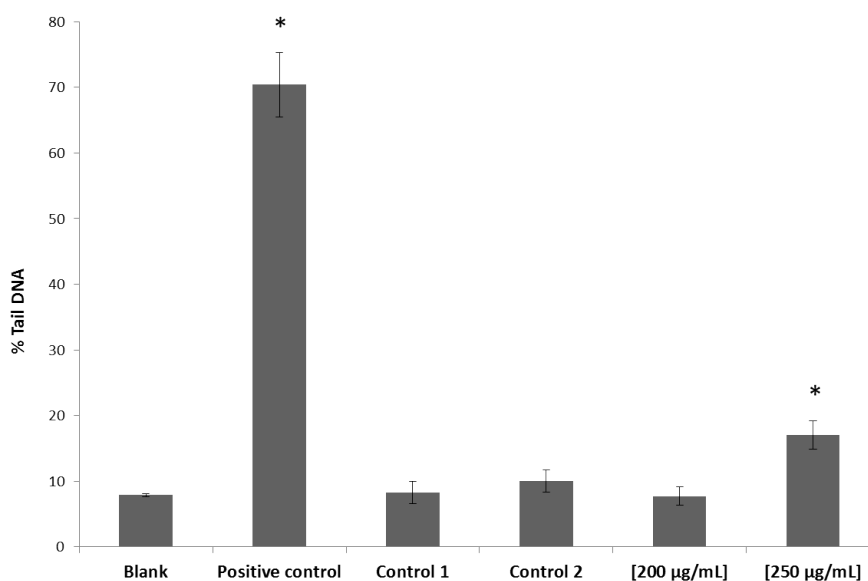


Fig. 6. Quantification of the % of Tail DNA representative of DNA damage induced in MCF-7 cells treated with a phenolic extract from *Leccinum vulpinum* Watling. Cells were treated with 200 µg/mL and 250 µg/mL of the studied phenolic extract for 24 h. Appropriate controls were included: untreated cells (Blank), cells treated with the positive control (H₂O₂, 1 mM) and cells treated with the corresponding volumes of the extract solvent (Control 1 and Control 2).

Overall, the phenolic extract from *L. vulpinum* seems to affect the human MCF-7 breast cancer cell line by causing DNA damage, which then probably caused a decrease in cellular proliferation and an increase in cell death by apoptosis.

Indeed, the results obtained by flow cytometry (as well as fluorescence microscopy) were conclusive and reported an increase in the percentage of MCF-7 cells undergoing programmed cell death following treatment with the studied phenolic extract. Moreover, it is well known that PARP is involved in the repair of DNA damage, being cleaved during apoptosis. In fact, cleavage of PARP by caspases is considered to be a hallmark of apoptosis (Chaitanya et al., 2010; Yang et al., 2004). Therefore, our findings clearly suggest the induction of apoptosis by this extract. The decrease in procaspase-9 levels following treatment means that caspase-9 was activated. This protease is known to then activate the downstream caspases-3 and/or -7, which in turn cleave PARP (Ferguson et al., 2003).

Additionally, even though the results obtained for *p*-H2AX have not been conclusive, the Comet assay proved that the extract induced DNA damage of MCF-7 cells.

4. Conclusions

This work was the first to report the bioactivity of *L. vulpinum* phenolic extract. This extract is characterised by the presence of hydroxybenzoic acids (gallic, protocatechuic and *p*-hydroxybenzoic acids) and the related compound cinnamic acid.

This mushroom extract seems to inhibit the growth of the human MCF-7 breast cancer cell line, by inducing DNA damage, inhibiting cellular proliferation and leading to cell death by apoptosis. This extract also revealed cytotoxic effects in the human breast non-malignant cell line, but at much higher concentrations.

This work has provided further knowledge on the potential of *L. vulpinum* phenolic extract as a source of bioactive compounds with antitumour potential.

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