Wild mushrooms *Clitocybe alexandri* and *Lepista inversa*: In vitro antioxidant activity and growth inhibition of human tumour cell lines

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Abstract

The *in vitro* antioxidant and growth inhibitory activity of extracts obtained from two Portuguese wild mushrooms (*Clitocybe alexandri* and *Lepista inversa*) was studied in human tumour cell lines. The extracts were phenolic (methanolic and ethanolic) and polysaccharidic (boiling water). The antioxidant activity assays included evaluation of radical scavenging capacity, reducing power and inhibition of lipid peroxidation measured in liposome solutions. Extract-induced cell growth inhibition was measured in four different tumour cell lines (lung, breast, colon and gastric cancer) using the SRB assay. The polysaccharidic extract of *Lepista inversa* was the most potent as antioxidant (EC_{50} < 1.8 ± 0.1 mg/ml), while the phenolic ethanolic extract of *Clitocybe alexandri* was the most potent as inhibitor of growth of the studied cancer cell lines (GI_{50} < 26.0 ± 1.3 μg/ml). Together, these activities indicate that these mushrooms are promising sources of bioactive compounds.

Keywords: Wild mushrooms; Antioxidant activity; Growth inhibitory activity.

1. Introduction

Edible mushrooms have been widely used as human food for centuries and have been appreciated for texture and flavour as well as some medicinal and tonic attributes. However, the awareness of mushrooms as being a healthy food and as an important source of biological active substances with medicinal value has only recently emerged. Various activities of mushrooms have been studied which include antibacterial, antifungal, antioxidant, antiviral, antitumour, cytostatic, immunosuppressive, antiallergic, antiatherogenic hypoglycemic, anti-inflammatory and hepatoprotective activities (Lindequist et al., 2005).

Many studies have concluded that edible mushrooms possess potent antioxidants. The antioxidants found in mushrooms are mainly phenolic compounds (phenolic acids and flavonoids), followed by tocopherols, ascorbic acid and carotenoids. These molecules were quantified in many different species mainly from Finland, India, Korea, Poland, Portugal, Taiwan and Turkey (Ferreira et al., 2009). Other phytochemicals have been isolated from medicinal mushrooms and three of these, which are cytostatic polysaccharide drugs, have been developed from mushrooms in Japan. These are "Krestin" (PSK), from the cultured mycelium of Kawaratake (*Trametes versicolor*), "Lentinan" from the fruiting bodies of Shiitake (*Lentinus edodes*) and "Schizophyllan" (Sonifilan) from the culture fluid of Suehirotake (*Schizophyllum commune*) (Mizuno, 1993). Lentinan and Schizophyllan are pure β-glucans, whereas PSK is a protein bound polysaccharide (Larone, 2002). The biological activity of these three products is related to their immunomodulating properties, which enhance the body's defences against various forms of infectious disease. These immunopotentiators (or immunoinitiators) are also referred as "biological response modifiers" (Zjawiony, 2004; Zaidman et al., 2005).

The most common causes of cancer related deaths in Europe in 2006 were lung, breast, colorectal and prostate cancer (Ferlay et al., 2007; Karim-Kos et al., 2008). Furthermore, evidence-based studies suggest there is a relationship between the physiopathology of several chronic diseases (*e.g.* cancer) and oxidative stress. Therefore, the use of foods with antioxidant capacity as phytochemical protectors may be relevant for the prevention of oxidative stress related diseases (Finco et al., 2009). Moreover, there is a need to discover new molecules that are able to effectively reduce tumour cell growth. In the present work we studied whether two Portuguese mushroom species possess antioxidant activities and/or tumour cell growth inhibition properties.

2. Materials and methods

2.1. Mushroom species

Samples of *Clitocybe alexandri* (Gillet) Konrad (*Tricholomataceae*) and *Lepista inversa* (Scop.: Fr.) Pat. (*Tricholomataceae*) were collected under Q*uercus pyrenaica* Willd. and mixed stands of *Quercus sp. and Pinus sylvestris* Ait., in Bragança (Northeast Portugal), in autumn 2008. Taxonomic identification of sporocarps was made according to several authors (Marchand, 1971-1986; Breitenbach and Kränzlin, 1984-2000; Bon, 1988; Courtecuisse, 1999; Courtecuisse and Duhem, 2005; online keys: http://www.mycokey.com/), and representative voucher specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança. Both species are saprotrophic and edible. All the samples were lyophilised (Ly-8-FM-ULE, Snijders) and reduced to a fine dried powder (20 mesh).

2.2. Standards and reagents

Methanol and ethanol were of analytical grade purity and supplied by Pronalab (Lisbon, Portugal). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA). Fetal bovine serum (FBS) was obtained from Invitrogen (Paisley, UK) and RPMI-1640 medium from Lonza (Basel, Switzerland). Dimethylsulfoxide (DMSO), sulforhodamine B (SRB) and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated.

2.3. Preparation of phenolic (methanolic and ethanolic) and polysaccharidic (boiling water) extracts

For methanolic extracts, each mushroom lyophilized sample (~2 g) was extracted with methanol (50 ml) mixture at -20 °C for 6 h. The extract was sonicated for 15 min, centrifuged at 4000 × *g* for 10 min, and filtered through Whatman nº 4 paper. The residue was then extracted with three additional 50 ml portions of methanol. The combined extracts were evaporated at 40 ºC to dryness and re-dissolved in DMSO.

The ethanolic and polysaccharidic (boiling water) extracts were prepared following the procedure described by Cheng et al. (2008) with some modification. Polysaccharides were extracted from lyophilized mushrooms (~1.5 g) with water at boiling temperature (50 ml) for 2 h and agitated (150 rpm; Velp Are magnetic stirrer) and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with two more portions of boiling water, in a total of 6 h of extraction. The combined extracts were lyophilized, and then 95 % ethanol (10 ml) was added and polysaccharides were precipitated overnight at 4 ºC. The precipitated polysaccharides were collected after centrifugation (Centorion K24OR- 2003 refrigerated centrifuge) at 3100 × *g* for 40 min followed by filtration, and then were lyophilized, resulting in a crude polysaccharidic sample. The ethanolic supernatant was evaporated at 40 ºC under reduced pressure

(rotary evaporator Büchi R-210), giving the ethanolic extract. The crude polysaccharidic samples were re-dissolved in water, while the ethanolic extracts were re-dissolved in ethanol for the antioxidant activity assays. For the tumour cell growth screening assays, both extracts were re-dissolved in DMSO. The solutions were stored at -20 ºC until further use. Final concentration of DMSO showed no interference with the biological activity tested.

2.4. Antioxidant activity

Phenolics determination. For the determination of the content in phenols in the ethanolic extracts, the sample (1 ml) was mixed with Folin-Ciocalteu phenol reagent (1 ml). After 3 min, saturated sodium carbonate solution (1 ml) was added to the mixture and adjusted to 10 ml with distilled water. The reaction was kept in the dark for 90 min (following Heleno et al., 2010), after which the absorbance was read at 725 nm (Analytikijena 200-2004 spectrophotometer). Gallic acid was used to calculate the standard curve of absorbance vs. concentration (1×10^{-5}-4×10^{-4} mol·l^{-1}; Y = 2.8557X–0.0021; R^2 = 0.9999) and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

DPPH radical-scavenging activity. This methodology was performed using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc), according to Heleno et al. (2010). The reaction mixture in each one of the 96-wells consisted of one of the serial concentrations of the extracts (30 µl) and aqueous methanolic solution (80:20 v/v, 270 µl) containing DPPH radicals (6×10^{-5} mol.l^{-1}). The mixture was left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH

discolouration using the equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50 % of radicals scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against extract concentration. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as standard.

Reducing power. The serial concentrations of the extracts (0.5 ml) were mixed with sodium phosphate buffer (200 $mmol.l^{-1}$, pH 6.6, 0.5 ml) and potassium ferricyanide (1 % w/v, 0.5 ml). The mixture was incubated at 50 ºC for 20 min, and trichloroacetic acid (10 % w/v, 0.5 ml) was added. The mixture (0.8 ml) was poured in the 48-wells, as also deionised water (0.8 ml) and ferric chloride (0.1 % w/v, 0.16 ml), and the absorbance was measured at 690 nm in the Microplate Reader described above (Heleno et al., 2010). The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 690 nm against extract concentration. Trolox was used as standard.

Inhibition of β-carotene bleaching. The antioxidant activity of the extracts was evaluated by the β-carotene linoleate model system. A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 ml). Two millilitres of this solution were added to a 100 ml round-bottom flask. After the chloroform was removed at 40 ºC under vacuum, linoleic acid (40 mg), Tween® 80 emulsifier (400 mg), and distilled water (100 ml) were added to the flask and vigorously shaken. Aliquots (4.8 ml) of this emulsion were transferred into different test tubes containing serial concentrations (0.2 ml) of the extracts. The tubes were shaken and incubated at 50 ºC in

a water bath (Heleno et al., 2010). As soon as the emulsion was added to each tube, the zero time absorbance at 470 nm was measured. A blank, devoid of β-carotene, was used for background subtraction. Lipid peroxidation inhibition was calculated using the following equation: (β-carotene content after 2 h of assay)/(initial β-carotene content) × 100. The extract concentration providing 50 % antioxidant activity (EC_{50}) was calculated by interpolation from the graph of antioxidant activity percentage against extract concentration. Trolox was used as standard.

2.5. Growth inhibition of Tumour Cell Lines

The effects of the extracts on the growth of human tumour cell lines was evaluated according to the procedure adopted in the NCI's *in vitro* anticancer drug screening, which uses SRB assay to assess cell growth inhibition (Skehan et al., 1990). This colorimetric assay estimates cell number indirectly, by staining cellular protein with the protein-binding dye SRB. Four human tumour cell lines were used: NCI-H460 (lung cancer), MCF-7 (breast cancer), HCT-15 (colon cancer) and AGS (gastric cancer). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 5 % heat-inactivated FBS at 37 ºC, in a humidified air incubator containing 5 % CO_2. Each cell line was plated at an appropriate density (5.0×10^3 cells/well for NCI-H460 and MCF-7, 1.0×10^4 cells/well for HCT-15 and 7.5×10^3 cells/well for AGS) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48 h with various extract concentrations. Following this incubation period, the adherent cells were fixed with 10 % trichloroacetic acid (final concentration), washed with 1 % acetic acid and stained with SRB. The bound stain was solubilised with 10 mM Tris and the absorbance was measured at 490 nm in a microplate reader (BIORAD 680 model). The concentration that inhibited growth in 50 % (GI_{50}) was calculated as described by

Monks et al. (1991). The growth inhibitory activity of the extracts was inferred from the SRB assay by comparing the absorbance of the wells containing extract-treated cells with the absorbance of the wells containing untreated cells, 48 h following treatment with the extracts, and subsequently comparing these results with the ones obtained for cells that had been fixed at time zero (time at which extracts were added) (Pedro et al., 2002).

2.6. Statistical analysis

For each assay 3-6 independent experiments were performed in duplicate. The results are expressed as mean values ± standard deviation (SD) or standard error (SE). One-way analysis of variance (ANOVA) followed by Tukey's HSD Test was performed with the SPSS v.16.0 software. Differences in p values bellow 0.05 were considered statistically significant.

3. Results and discussion

In the present work we tested the whole mushrooms extracts, taking advantage of the idea that complex mixture of phytochemicals may have potential additive or synergistic effects (Liu, 2004). Three different extracting solvents were used in order to obtain low molecular weight compounds such as phenolic antioxidants (methanol and ethanol) and high molecular weight compounds such as polysaccharides (boiling water).

Results show that the ethanolic extracts of *Lepista inversa* (10.8 mg GAEs/g) and *Clitocybe alexandri* (6.3 mg GAEs/g) have higher phenolic content than the corresponding methanolic extracts (**Table 1**). The phenolic content found in all the extracts of *L. inversa* was significantly (p<0.05) higher than what was found in *C. alexandri*. We hypothesize that this could be related to differences in the lignocelulolitic

capacities of the two saprotrophic species, which are well known to vary along the fructification process, but this needs further confirmation. Many mushroom species have the biosynthetic potential to produce and secrete a wide spectrum of enzymes, both saccharifying (cellulases, hemicellulases, xylanases) and oxidative (lignin peroxidases, manganese peroxidases and lacases), which enable them to thrive over a range of plant wastes. The degraded products are used as their energy source to produce protein rich biomass (Periasamy and Natarajan, 2004). In previous studies some of us have reported that these mushrooms possess antioxidants other than phenols, such as tocopherols (Heleno et al., 2010) and reducing sugars (Heleno et al., 2009).

All the extracts proved to have free radical scavenging activity and reducing power, but to different extent. The antioxidant activity was measured against radical species generated in the reaction system, such as DPPH radicals (DPPH scavenging activity assay) and linoleate-free radical (β-carotene bleaching inhibition assay), or by the reducing effect on Fe^{3+}/ferricyanide complex (reducing power assay). Ethanolic extracts gave higher antioxidant activity (lower EC_{50} values; **Table 1**) than methanolic extracts, which is in agreement with the highest content in phenolics found in the first extracts. Nevertheless, it was the polysaccharidic extracts that revealed the most potent antioxidant activity in the three assays (EC_{50} < 2.5 mg/ml; T**able 1**).

The effects of the mushroom extracts on the growth of four human tumour cell lines (NCI-H460, MCF-7, HCT-15 and AGS), represented as the concentrations that were able to cause 50 % of cell growth inhibition (GI_{50}), are summarized in **Table 2**. Phenolic (methanolic and ethanolic) and polysaccharidic (boiling water) extracts from *Clitocybe alexandri* and *Lepista inversa* revealed capacity to inhibit 50 % of the growth of all the human tumour cell lines studied, with concentrations lower than 160 µg/ml. *C.*

alexandri was more potent than *L. inversa* in inhibiting the growth of the four studied human tumour cell lines. For *C. alexandri*, the lower GI_{50} values were generally obtained with the ethanolic extracts, while for *L. inversa*, methanolic extracts proved to be generally more potent, with the exception of the AGS cells (in which the more potent extract was the ethanolic). For both mushrooms, the polysaccharidic extract was the less potent one.

The growth inhibitory effect presented by the extracts, as determined by the SRB assay, results froma balance between cell proliferation and cell death. However, this assay can not infer which effect (cytotoxic or cytostatic) is more prevalent. Further assays would need to be carried out in order to clarify the mechanism of action of these extracts, *e.g.* BrdU (for proliferation) and Annexin V-FITC/PI (for apoptosis). Nonetheless, for the ethanolic extract of *Clitocybe alexandri,* where the % growth (in NCI-H460 cells) at high concentrations is lower than that observed at time zero, there is a clear indication that this extract is exerting a cytotoxic effect in addition to the cytostatic effect (**Figure 1**).

In summary, polysaccharidic extract of *Lepista inversa* was the most potent as antioxidant, while the ethanolic phenolic extract of *Clitocybe alexandri* was the most potent as inhibitor of growth of human tumour cell lines. This interesting growth inhibitory activity proves that these mushrooms, particularly the ethanolic extract of *Clitocybe alexandri*, are promising sources of bioactive compounds. Methanolic extracts of fresh fruiting bodies of *Lepista inversa* collected in France were described in the literature as having cytotoxic activity against two mouse tumour cell lines, L1210 (lymphocytic leukemia) and 3LL (Lewis lung carcinoma), and against four human tumour cell lines: K-562 (leukemia), U251 (glioma), DU145 (prostate cancer) and

MCF7 (breast cancer) (Bezivin et al., 2002). Clitocine, an exocyclic amino nucleoside isolated from this mushroom also revealed growth inhibitory activity against the same four tumour cell lines (Fortin et al., 2006). Nevertheless, there are no reports of growth inhibitory activity against lung, colon and gastric human cancer cells. To our knowledge, this is the first report describing growth inhibitory properties of *Clitocybe alexandri* in human tumour cell lines. Future work will elucidate the mechanism of action of these extracts leading to the observed cell growth inhibition.

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Table 1. Antioxidant activity of the mushroom extracts.

Species	Extracts	Extraction yield (%)	Phenolics (mg GAEs/g)	DPPH scavenging activity	Reducing power	β-carotene bleaching inhibition
Clitocybe alexandri	Phenolic (methanolic)[a]	47.7 ± 5.3	1.5 ± 0.1	28.7 ± 3.2	7.0 ± 0.4	4.5 ± 0.2
	Phenolic (ethanolic)	3.5 ± 0.2	6.3 ± 0.4	10.7 ± 0.8	2.3 ± 0.0	3.7 ± 0.1
	Polysaccharidic (boiling water)	30.3 ± 2.8	na	2.5 ± 0.0	0.9 ± 0.0	1.2 ± 0.0
Lepista inversa	Phenolic (methanolic)[a]	39.0 ± 1.9	3.6 ± 0.1	10.6 ± 1.1	2.9 ± 0.1	1.1 ± 0.1
	Phenolic (ethanolic)	4.6 ± 0.5	10.8 ± 0.7	9.3 ± 0.5	1.4 ± 0.1	1.5 ± 1.1
	Polysaccharidic (boiling water)	32.2 ± 3.1	na	1.8 ± 0.1	0.7 ± 0.0	0.9 ± 0.1

Results are expressed as EC_{50} (concentrations of extracts in mg/ml that cause 50 % of antioxidant activity, unless for reducing power that is 0.5 of absorbance), and show means ± SE of 3 independent experiments performed in duplicate; na- not analysed; [a] Heleno et al., 2010.

Table 2. Effect of the mushroom extracts on the growth of human tumour cell lines.

Species	Extracts	NCI-H460 (lung cancer)	MCF-7 (breast cancer)	HCT-15 (colon cancer)	AGS (gastric cancer)
Clitocybe alexandri	Phenolic (methanolic)	34.8 ± 2.8	34.2 ± 1.4	36.9 ± 3.1	36.1 ± 2.3
	Phenolic (ethanolic)	24.8 ± 2.3	17.9 ± 1.3	21.7 ± 2.3	26.0 ± 1.3
	Polysaccharidic (boiling water)	24.5 ± 1.8	46.8 ± 1.6	59.1 ± 0.7	51.7 ± 0.9
Lepista inversa	Phenolic (methanolic)	36.3 ± 5.1	45.2 ± 3.1	39.7± 4.6	67.4 ± 5.5
	Phenolic (ethanolic)	118.3± 2.5	79.1 ± 11.8	42.3 ± 4.5	58.5 ± 3.3
	Polysaccharidic (boiling water)	155.0± 3.5	137.4 ± 1.3	77.4 ± 5.5	99.9 ± 7.8

Results are expressed as GI_{50} (concentrations of extracts in μg/ml that cause 50 % of growth inhibition of human tumour cell lines), and show means ± SE of 3-6 independent experiments performed in duplicate.

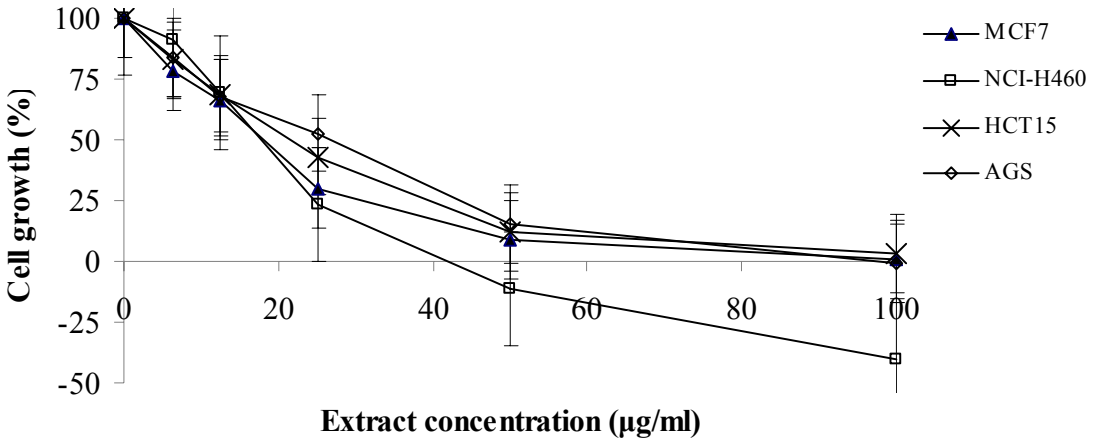

Figure 1. Percentage of cell growth of the ethanolic phenolic extract of *Clitocybe alexandri* against four human tumour cell lines (breast, MCF7; lung, NCI-H460; colon, HCT-15; gastric, AGS) compared to control. Results are the mean ± SD of at least three independent experiments, performed in duplicate.