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Suillus luteus methanolic extract inhibits cell growth and proliferation of a colon cancer cell line

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ABSTRACT

Several edible mushrooms extracts are known to have tumor cell growth inhibitory potential. The objective of this work was to study this potential in extracts of *Suillus luteus* collected from the Northeast of Portugal. Various extracts were prepared and their effect on tumor cell growth was studied with the SRB assay in four human tumor cell lines: MCF-7 (breast), NCI-H460 (non-small cell lung cancer), AGS (gastric) and HCT-15 (colon). The methanolic extract of *S. luteus* was the most potent extract. This extract was slightly more potent in the HCT-15 cells (with mutant p53, $GI_{50} = 17.8 \pm 1.6$ μg/mL) than in the other cell lines tested, which suggested that its effect was not p53-dependent. Indeed, in HCT-15 cells, an increase in the levels of p53 was detected but no alterations in some of the proteins transactivated by p53 (e.g. p21 or Bax) were found. The extract caused an increase in the cellular levels of p-H2A.X, which is suggestive of DNA damage. Growth inhibition in these cells was mostly due to inhibition of cell proliferation and an increase in the % of cell in the G1 phase of the cell cycle. An increase in cell death was also found, even though to very low levels. In addition, this extract was not cytotoxic to primary cultures of porcine hepatocytes ($GI_{50} > 400$ μg/mL). Together, these results indicate that the *S. luteus* methanolic extract may be an interesting source of compounds that inhibit the proliferation of tumor cells but further studies should be carried out in order to understand its potential.

1. Introduction

The majority of new drugs have been generated from either natural products (secondary metabolites) or analogs inspired by them. Many drugs such as antibiotics (penicillin, tetracycline and erythromycin), antiparasitics (avermectin), antimalarials (quinine, artemisinin), lipid control agents (lovastatin and analogs), immunosuppressants for organ transplants (cyclosporin, rapamycins) or anticancer drugs (taxol, doxorubicin) have been found or produced in this way (Li & Vederas, 2009).

Mushrooms (macrofungi) contain a vast diversity of biomolecules with nutritional (Kalač, 2009) and/or medicinal properties (Poucheret, Fons, & Rapior, 2006). They have been recognized as functional foods and as a source of compounds for the development of nutraceuticals or medicines, including compounds with antitumor properties (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007; Zaidman, Yassin, Mahajna, & Wasser, 2005).

The most studied compounds are high-molecular-weight ones, such as the β-glucans "Lentinan" from the fruiting bodies of *Lentinus edodes*, "Schizophyllan" from the culture fluid of *Schizophyllum commune*, or the polysaccharopeptides PSP and "Krestin" (PSK) from the cultured mycelium of *Coriolus versicolor* (Ferreira, Vaz, Vasconcelos, & Martins, 2010; Luk et al., 2011). Despite their commercialization, limited clinical studies in cancer patients have been conducted. In addition to the mentioned well-known medicinal mushrooms, previous work from our research group showed that other wild unexplored species from Portugal are notable for their promising antitumor potential, such as *Clitocybe alexandri* and *Suillus collinitus*. Indeed, their phenolic extracts (including low molecular weight compounds) showed effects on cell cycle and induced apoptosis in human lung and breast cancer cell lines, respectively (Vaz, Almeida, Ferreira, Martins, & Vasconcelos, 2012; Vaz, Ferreira, et al., 2012).

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Following our research in wild edible species from Northeast Portugal, the present work describes the cytotoxic activity of methanolic, ethanolic and boiled water extracts of *Suillus luteus* in various human tumor cell lines: lung, breast, colon and gastric cancer. The extract with the highest cell growth inhibitory activity (methanolic extract) was chosen to be further investigated regarding its possible mechanism of action in the most susceptible cell line studied (colon cancer), by evaluation of its effect on the cell cycle profile, cellular proliferation and apoptosis. The chemical characterization of *S. luteus* was previously reported by our research group. This mushroom presented protocatechuic acid (0.47 mg/100 g dry weight), cinnamic acid (0.41 mg/100), α-tocopherol (19.14 μg/100 g), β-tocopherol (15.34 μg/100 g), γ-tocopherol (366.77 μg/100 g), δ-tocopherol (78.51 μg/100 g), mannitol (1.29 g/100 g), trehalose (1.35 g/100 g), polyunsaturated fatty acids (52.75%, with linoleic acid – 52.31% – as the main fatty acid), monounsaturated fatty acids (32.93%, with oleic acid – 31.24% – as the main fatty acid), and saturated fatty acids (14.32%, with palmitic acid — 10.57% as the main fatty acid) (Reis et al., 2011).

2. Materials and methods

2.1. Sample collection and preparation of the extracts

Samples of *S. luteus* (L.: Fries) Gray (edible mushroom) were collected in Bragança (Northeast Portugal), in the autumn of 2009. Taxonomic identification of sporocarps was made according to Courtecuisse and Duhem (2005) and representative voucher specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança. The samples were lyophilized (Ly-8-FM-ULE, Snijders, Holland) and reduced to a fine powder (20 mesh).

Enriched phenolic (methanolic and ethanolic) and polysaccharidic (boiled water) extracts were prepared from the lyophilized powder following the procedure previously described by us (Vaz et al., 2010). Briefly, to obtain the methanolic extract, the lyophilized sample (~2 g) was extracted twice 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, filtered through Whatman No. 4 paper and concentrated under reduced pressure (rotary evaporator Büchi R-210). To obtain the polysaccharidic (boiling water) extract, the lyophilized sample (~1.5 g) was extracted three times with water at boiling temperature (50 mL) for 2 h and subsequently filtered through Whatman No. 4 paper. The combined extracts were lyophilized, and then 95% ethanol (10 mL) was added and polysaccharides were precipitated overnight at 4 °C. 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 concentrated under reduced pressure, giving the ethanolic extract.

For the subsequent assays, the extracts were re-dissolved in DMSO and the solutions were stored at −20 °C until further use.

2.2. Cell culture

2.2.1. Human tumor cell lines

The cell lines used were: NCI-H460 (lung cancer), AGS (gastric cancer), MCF-7 (breast cancer) and HCT-15 (colon cancer). Cells were plated in RPMI 1640 medium supplemented with 5% heat-inactivated FBS (Lonza). All cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2.2. Primary cells of porcine liver

A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, and it was designed as PLP2. Briefly, the liver tissues were rinsed in Hank's balanced salt solution containing 100 U/mL penicillin and 100 μg/mL streptomycin and divided into 1 × 1 mm^3 explants. Some of these explants were placed in 25 cm^2 tissue flasks in DMEM medium supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids and 100 U/mL penicillin and 100 mg/mL streptomycin and incubated at 37 °C with a humidified atmosphere containing 5% CO_2. The medium was changed every two days. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. The cells were cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin (Abreu et al., 2011).

Table 1
Growth inhibitory activity of different extracts of *S. luteus* on human tumor cell lines and on primary cells of porcine liver.

	Tumor cell lines			Primary cells	
	NCI-H460	HCT-15	MCF-7	AGS	PLP2
Boiled water	>400	>275[a]	>400	>400	>400
Methanolic	30.33 ± 1.1	17.75 ± 1.6	32.25 ± 5.7	30.30 ± 3.1	>400
Ethanolic	>400	>400	>400	>400	>400

Results are expressed as GI_{50} (concentration of extract in μg/mL that causes 50% of cell growth inhibition), and show means ± SD of 3 independent experiments performed in duplicate.

[a] The GI_{50} concentration could not be determined but was always superior to 275 μg/mL.

2.3. Analysis of cell growth

The effects of the extracts on the growth of human tumor cell lines were evaluated, according to the procedure adopted in the NCI's in vitro anticancer drug screening (Skehan et al., 1990). 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, and 1.0 × 10^4 cells/well for PLP2) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48 h with various extract concentrations. The DMSO concentrations used presented no growth inhibitory effect in these cell lines.

Following this incubation period, the adherent cells were fixed with 10% trichloroacetic acid (final concentration), stained with SRB and excess dye washed with 1% acetic acid. The bound SRB was solubilized with 10 mM Tris and the absorbance was measured at 510 nm in a microplate reader (Multi-mode microplate Synergy™ MX, BioTek) and analyzed with the Gen5™ software (BioTek). The concentration that inhibited growth in 50% (GI_{50}) was calculated according to Monks et al. (1991) and as previously described by the authors (Vaz et al., 2010).

2.4. Cell cycle analysis

For the analysis of cell cycle phase distribution, HCT-15 cells were plated at 2 × 10^5 cells/well in 6-well plates and incubated for 24 h. Cells were then incubated with complete medium (blank), with *S. luteus* methanolic extract at the GI_{50} and 2 × GI_{50} concentrations or with DMSO (control). Cells were harvested following a 24 and 48 h of incubation period with the extract, further fixed in 70% ethanol and kept at 4 °C until analysis. Prior to analysis, cells were incubated with Propidium Iodide (5 μg/mL) and RNase A in PBS (100 μg/mL) for 30 min on ice (Vaz, Almeida, et al., 2012).

Cellular DNA content was analyzed at the Advanced Flow Cytometry Unit (IBMC/INEB) using a FACS Calibur (BD) flow cytometer. Cell cycle profile was subsequently analyzed using the FlowJo 7.2 software (Queiroz et al., 2010).

2.5. Programmed cell death (TUNEL) and proliferation (BrdU) assays

For the TUNEL and BrdU assays, cells were plated, respectively at 7.5 × 10^4 per well in 12-well plates, and 2 × 10^5 per well in 6-well plates, and incubated for 24 h. Cells were then further incubated