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Wild mushrooms and their mycelia as sources of bioactive compounds: Antioxidant, anti-inflammatory and cytotoxic properties

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ABSTRACT

Mushrooms are important sources of natural bioactive compounds. *Pleurotus eryngii* (DC.) Quél is recognized for its organoleptic quality and health effects, being extensively commercialized. Instead, *Suillus bellinii* (Inzenga) Watling is an ectomycorrhizal symbiont, whose main properties were scarcely reported. Considering current trends, the mycelia and the culture media of these mushrooms might be potential sources of bioactive compounds. Accordingly, *P. eryngii* and *S. bellinii* were studied for their phenolic acids and sterols, antioxidant capacity, anti-inflammatory effect and anti-proliferative activity. *S. bellinii* mycelia showed higher contents of ergosterol and phenolic compounds (also higher in its fruiting body) and stronger antioxidant activity than *P. eryngii*. Conversely, *P. eryngii* mycelia showed anti-inflammatory (absent in *S. bellinii* mycelia) and a cytotoxicity similar (sometimes superior) to its fruiting bodies, contrarily to *S. bellinii*. Furthermore, the assayed species showed differences in the growth rate and produced mycelia, which should be considered in further applications.

1. Introduction

Since ancient times, there is a wide interest in using edible mushrooms as functional foods or sources of natural medicine components (Shi, Zhao, Jiao, Shi, & Yang, 2013).

Pleurotus eryngii is one of the most favored and widely consumed mushroom species, being also called "king oyster mushroom". Besides its organoleptic quality, *P. eryngii* is acknowledged as a good dietary source of bioactive compounds with reported effects on the reduction of hyperlipidemia (Chen et al., 2012), tumor growth, hepatogenic and atherosclerotic conditions (Chen et al., 2014; Yang et al., 2013). Most of the available reports describe the polysaccharide fraction of *P. eryngii*, which was highlighted as having antioxidant, anti-aging, anti-tumor and hepatoprotective activity (Gan, Ma, Jiang, Wang, & Zeng, 2012; Jayakumar, Sakthivel, Thomas, & Geraldine, 2008). Nevertheless, other important bioactive compounds such as polyphenols, peptides, sterols and dietary fiber were also reported in this species (Chen et al., 2012; Ferreira, Barros, & Abreu, 2009; Mishra et al., 2013).

The genus *Suillus* includes several C-demanding species, such as *Suillus bellinii* (Inzenga) Watling, which are able to produce large amounts of biomass and exudates (Izumi, Elfstrand, & Fransson, 2013). *S. bellinii* is an ectomycorrhizal symbiont, which might grow associated with a wide range of plant hosts (Franco & Castro, 2015). Nevertheless, there are very scarce data on the chemical composition and bioactivity of this mushroom, having been reported only for its organic acids, phenolic acids (Ribeiro et al., 2006), alcohols (Guedes De Pinho et al., 2008) and antimicrobial activity (Dulger, Hacioglu, & Suerdem, 2006).

Besides the fruiting bodies, the mycelia and the culture media utilized in mushroom cultivation have also been explored as potential sources of bioactive compounds (Ma et al., 2016). The cultured mycelia are becoming a promising alternative as a source of fungal bioactive compounds, mostly due to the shorter incubation time and easier culture conditions (less required space, low probability of contamination and higher production of biomass, when compared to the fruiting bodies) (Gan et al., 2012; Zhang et al., 2016).

Considering the species herein, the fruiting bodies of *P. eryngii* were previously studied for their chemical composition,

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antioxidant and anti-inflammatory activities, after being harvested at different periods (Barreira, Oliveira, & Ferreira, 2014; Lin et al., 2014; Reis, Barros, Sousa, Martins, & Ferreira, 2014; Reis, Martins, Barros, & Ferreira, 2012). However, as far as we know, the anti-inflammatory activity of *S. bellinii* was not reported yet. In addition, the culture media and mycelia of both species were never studied regarding the anti-tumoral and anti-inflammatory activities. Accordingly, their composition in phenolic acids and sterols, antioxidant capacity (scavenging DPPH radicals, reducing power, β-carotene bleaching inhibition and TBARS formation inhibition), as well as their anti-proliferative activity (using MCF-7, NCI-H460, HeLa and HepG2 cell lines) and anti-inflammatory effect (by down-regulating LPS-stimulated NO in RAW264.7 cells) were evaluated.

With this study it was mainly intended to evaluate the effects of different culture conditions on the phenolic acids and ergosterol profiles and in the bioactivity of both species, to fully characterize their potential use in food or pharmaceutical applications.

2. Materials and methods

2.1. Wild samples and in vitro production of mycelia

Two species of wild mushrooms, *Pleurotus eryngii* (DC.) Quél and *Suillus belinii* (Inzenga) Watling, were collected in Bragança (Northeast Portugal) during November 2015. Mycelium was isolated from sporocarps of each sample on different solid: i) potato dextrose agar medium (PDA) (Biolab); ii) Melin-Norkans incomplete medium (without micronutrients, casaminoacids and malt extract) (iMMN solid), and liquid: i) potato dextrose broth (PDB); ii) Melin-Norkans incomplete (without micronutrients, casaminoacids and malt extract) (iMMN liquid) culture media (Marx, 1969).

Mycelia were grown in Petri dishes with 10 mL of solid media and flasks with 20 mL of liquid media. Petri dishes and flasks were placed at 22 °C in the dark until mycelium covered most of the medium: 21 days for *P. eryngii* and 42 days for *S. bellinii*, approximately. Radial growth measurements were registered every week from the inoculation time until the full growth of the mycelium (covering all available area). The mycelia were further recovered from the medium.

Fruiting bodies, mycelia and culture media were lyophilized (FreeZone 4.5, Labconco, MO, USA) and ground to a fine powder (20 mesh) and weighted to obtain the dry biomass (dw).

2.2. Standards and reagents

Acetonitrile and methanol of high-performance liquid chromatography (HPLC) grade were obtained from Lab-Scan (Lisbon, Portugal). Trolox (6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid), ergosterol and phenolic acids standards, sulforhodamine B, trypan blue, trichloroacetic acid (TCA), tris lipopolysaccharide (LPS) and dexamethasone were purchased from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Dulbecco's modified Eagle's medium (HyClone), Hank's balanced salt solution (HBSS) and all the additional culture media components were purchased from Gibco Invitrogen Life Technologies (Paisley, UK). RAW264.7 cells were acquired from ECACC ("European Collection of Animal Cell Culture") (Salisburg, UK), Griess reagent system kit was purchased from Promega, thiamine, casamino acids, malt extract and agar were obtained from Panreac AppliChem (Barcelona, Spain). The microbial culture media were acquired from Oxoid microbiology products (Hampshire, United Kingdom). Other reagents and solvents (analytical grade) were obtained from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Preparation of the extracts

The extraction was carried out by stirring the samples (≈2 g) with methanol (30 mL) at 25 °C and 150 rpm, for 1 h. The extract was separated from the residue by filtration through Whatman No. 4 paper to a round flask. The residue was re-extracted once more under the same conditions and the filtrates were combined and concentrated under vacuum (rotary evaporator, Büchi, Flawil, Switzerland) (Reis et al., 2012).

2.4. Chemical characterization of the extracts

2.4.1. Analysis of phenolic acids

The purified extracts were prepared in methanol (20 mg/mL), filtered through a 0.22 μm nylon syringe filter and further analyzed by ultra-fast liquid chromatography (UFLC) using a Shimadzu 20A series (Shimadzu Cooperation, Kyoto, Japan) (Reis et al., 2012). The quantification (μg/g of extract) was made by comparing the area of chromatographic peaks (280 and 320 nm) with the calibration curves (5–100 μg/mL) of the corresponding commercial standards: protocatechuic acid (y = 164741x, R^2 = 0.9996), *p*-hydroxybenzoic acid (y = 113523x, R^2 = 0.9993), *p*-coumaric acid (y = 433521x, R^2 = 0.9981) and cinnamic acid (y = 583527x, R^2 = 0.9961), 5–80 μg/mL.

2.4.2. Analysis of ergosterol

The sterol extracts were dissolved in methanol (20 mg/mL), filtered through a 0.22 μm nylon syringe filter and characterized by high performance liquid chromatography coupled to an ultraviolet detector (HPLC-UV) (Barreira et al., 2014). Chromatographic data (obtained at 285 nm) were analyzed using Clarity 2.4 Software (DataApex, Podohradska, Czech Republic). Ergosterol was quantified (mg/g of extract) using the internal standard (cholecalciferol) method.

2.5. Evaluation of bioactive properties

2.5.1. Antioxidant activity

The final extracts were dissolved in methanol at appropriate concentrations (10–80 mg/mL) and several dilutions were obtained from the stock solutions: 0.005–50 mg/mL, depending on the assay.

The sample concentrations providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene/linoleate and TBARS assays) or absorbance at 690 nm (reducing power assay) against sample concentrations (Heleno, Barros, Sousa, Martins, & Ferreira, 2010). Trolox was used as standard.

2.5.2. Antioxidant activity

DPPH radical scavenging activity. This methodology was performed using an ELX800 Microplate Reader (Bio-Tek). The reaction mixture in each one of the 96-wells consisted of one of the different concentrations of the extracts (30 μL) and methanolic solution (270 μL) containing DPPH radicals (6×10^{-5} mol/L). 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 discoloration 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.