Food Chemistry 230 (2017) 40–48

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

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

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ARTICLE INFO

Article history:
Received 13 September 2016
Received in revised form 1 March 2017
Accepted 6 March 2017
Available online 7 March 2017

Keywords:
Pleurotus eryngii
Suillus bellinii
Ergosterol
Phenolic acids
Antioxidant activity
Anti-inflammatory activity
Anti-proliferative activity

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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http://dx.doi.org/10.1016/j.foodchem.2017.03.026
0308-8146/

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.

Reducing power. Was performed using the Microplate Reader described above. The different concentrations of the extracts (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). For each concentration, the mixture was incubated at 50 °C 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 deionized water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm.

Inhibition of β-carotene bleaching. β-carotene (2 mg) was dissolved in chloroform (10 mL) and 2 mL of this solution were pipetted into a round-bottom flask. After the chloroform was removed at 40 °C under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing different concentrations of the extracts (0.2 mL). The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. β-Carotene bleaching inhibition was calculated using the following equation: (absorbance after 2 h of assay/initial absorbance) × 100.

TBARS (thiobarbituric acid reactive substances) assay. Porcine brains were obtained from official slaughtered animals, dissected, and homogenized with a Polytron in ice cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 w/v brain tissue homogenate which was centrifuged at 3000*g* for 10 min. An aliquot (100 μL) of the supernatant was incubated with the different concentrations of the samples solutions (200 μL) in the presence of $FeSO_4$ (10 mM; 100 μL) and ascorbic acid (0.1 mM; 100 μL) at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 500 μL), followed by thiobarbituric acid (TBA, 2%, w/v, 380 μL), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000*g* for 10 min to remove the precipitated protein, the color intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A − B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively.

2.5.3. Anti-inflammatory activity

Cells treatment. For the anti-inflammatory activity assay, the methanolic extracts were dissolved in water at a concentrated of 8 mg/mL. For the various assays, the extracts were then submitted to further dilutions from 0.4 mg/mL to 0.005 mg/mL. The mouse macrophage-like cell line RAW 264.7 was cultured in DMEM medium supplemented with 10% heat-inactivated foetal bovine serum, glutamine and antibiotics at 37 °C under 5% CO_2, in humidified air. For each experiment, cells were detached with a cell scraper. In the experiment cell density of 5×10^5 cells/mL was used, and the proportion of dead cells was less than 5% according to the Trypan blue dye exclusion test. Cells were seeded in 96-well plates at 150,000 cells/well and allowed do attach to the plate overnight. Subsequently, cells were treated with the various concentrations of each extract for 1 h. Dexamethasone (50 μM) was used as a positive control for the experiment. The following step was the stimulation with LPS (1 μg/mL) for 18 h. The effect of all the tested samples in the absence of LPS was also evaluated, in order to observe if they induced changes in Nitric oxide (NO) basal levels. In negative controls, no LPS was added. Both extracts and LPS were dissolved in supplemented DMEM (Souza et al., 2015).

Nitric oxide determination. For the determination of nitric oxide, a Griess Reagent System kit was used, which contains sulphanilamide, N-(1-napthyl)ethylenediamine hydrochloride (NED) and nitrite solutions. A reference curve of nitrite (sodium nitrite 100 μM to 1.6 μM; y = 0.0066x + 0.1349; R^2 = 0.9986) was prepared in a 96-well plate. The cell culture supernatant (100 μL) was transferred to the plate and mixed with sulphanilamide and NED solutions, 5–10 min each, at room temperature. The nitric oxide produced was determined by measuring the absorbance at 540 nm (microplate reader ELX800 Biotek), and by comparison with the standard calibration curve (Souza et al., 2015). The results were expressed in EC_{50} values (μg/mL), which correspond to the sample concentration providing 50% of inhibition of nitric oxide (NO) production.

2.5.4. Cytotoxic activity

The final extracts were dissolved in water at 8 mg/mL and several dilutions were obtained from the stock solutions: 0.125–0.4 mg/mL. Ellipticine was used as positive control.

2.5.4.1. In human tumor cell lines. MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma) were used as human tumor cell lines and the cell density determination was performed using a sulforhodamine B assay previously described by Guimarães et al. (2013). RPMI-1640 medium containing 10% heat-inactivated FBS and 2 mM glutamine was used to routinely maintain the adherent cell cultures at 37 °C, in a humidified air incubator containing 5% CO2. For the experiments, each cell line was placed at an appropriate density (1.0 × 104 cells/well) into 96-well plates. A procedure using the sulforhodamine B assay was performed. The extract concentration that inhibited 50% of the net cell growth (GI_{50}) was calculated from the graph of sample concentration against percentage of growth inhibition and expressed in μg/mL extract.

2.5.4.2. In non-tumor cells. The effect of the extracts on the growth of porcine liver primary cells (PLP2), established by the group, was evaluated by the sulforhodamine B colorimetric assay with some modifications as described by Abreu et al. (2011). Briefly, the liver tissue was rinsed in Hank's balanced salt solution containing 100 U/mL penicillin + 100 μg/mL streptomycin, and was divided into 1 × 1 mm^3 explants. Some of them were placed into 25 cm^2 tissue flasks containing DMEM medium (supplemented with 10% fetal bovine serum, 2 mM non-essential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin) and incubated at 37 °C under a humidified atmosphere with 5% CO_2. Phase contrast microscope was used for direct monitoring of the cell cultivation every 2–3 days. Before reaching the confluence, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in commercial DMEM medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The results were expressed as GI_{50} values (sample concentration that inhibited 50% of the net cell growth) in μg/mL extract.

2.6. Statistical analysis

Three independent samples of each culture component, fruiting body and fungal species were used. Data were expressed as mean ± standard deviation. All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., USA).

The results were compared through one-way ANOVA after checking the normal distribution of the residuals (Shapiro Wilk's test) and the homogeneity of variance (Levene's test). The dependent variables were classified using multiple comparison tests (Tukey's honestly significant difference (HSD) for homoscedastic distributions and Tamhane's T2 for the heteroscedastic ones).

3. Results and discussion

Edible mushrooms, in general, are esteemed primarily for their nutritional value and bioactive properties mostly provided by different active substances, such as polysaccharides, lipids, peptides, sterols, or fiber (Ren, Wang, Guo, Yuan, & Yang, 2016). The great majority of the studies reporting the previous features are conducted on the fruiting body, but the mycelia, as well as the culture media utilized in different stages of mushroom production, might also represent a good source of valuable compounds.

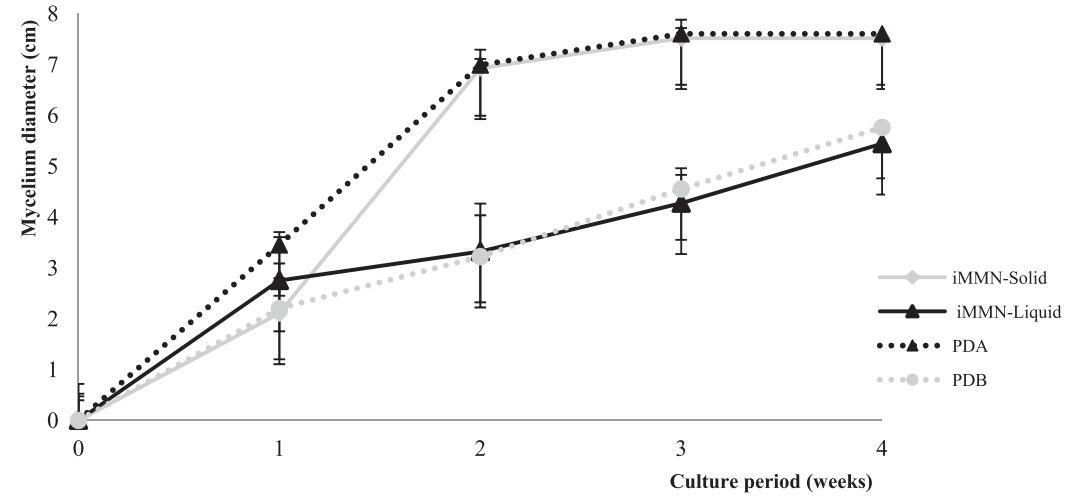
Besides the differences in bioactive compounds and corresponding activities, the growth rate and yielded biomass of mycelia are of paramount importance, since these parameters might define the industrial interest of each species. Accordingly, both indicators are presented in Figs. 1 and 2 to allow a proper assessment of their true applicability. As it can be seen, *P. eryngii* presented a higher growth rate, despite the similarities in the produced biomass (except for the mycelia grown in PDB).

In the following sections, the mycelia and culture media are compared by evaluating different bioactive compounds and biological activity indicators. In all tables, results corresponding to wild samples of each studied species are also presented as reference values.

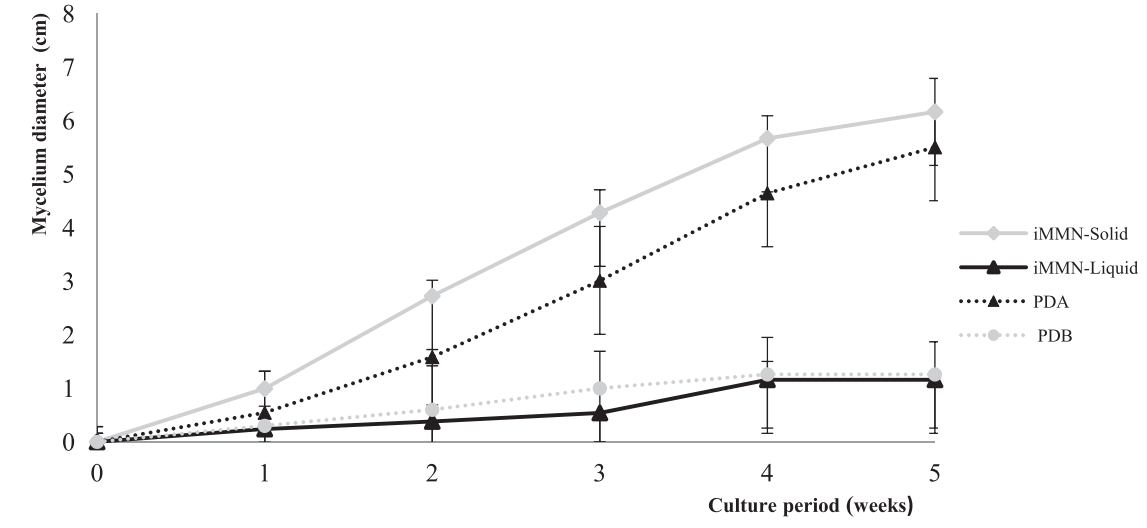
3.1. Chemical characterization of the extracts

The phenolic acids profile and ergosterol contents are presented in Table 1. In general, the fruiting body presented higher contents in phenolic acids (Fig. 3), but it was very interesting to find that the mycelium of *S. bellinii* (independently of the culture conditions) gave higher contents (8.9–12.4 mg/g extract) in ergosterol (Fig. 3) than the corresponding wild samples (6.5 mg/g extract). There are limited data on the sterols content of *S. bellinii*, but it was reported as having 12 mg/100 g fw ergosterol (Kalogeropoulos, Yanni, Koutrotsios, & Aloupi, 2013), which is higher than the values reported in the present work.

P. eryngii gave lower contents in ergosterol, either considering the mycelia (0.10–1.0 mg/g extract), as well as the fruiting body (4.1 mg/g extract), when compared to *S. bellinii*. The ergosterol content in *P. eryngii* was previously reported as 20 mg/100 g dw



(A)



(B)

Fig. 1. Growth in diameter of the mycelia of *P. eryngii* (A) and *S. bellinii* (B) cultivated in different culture media throughout time.

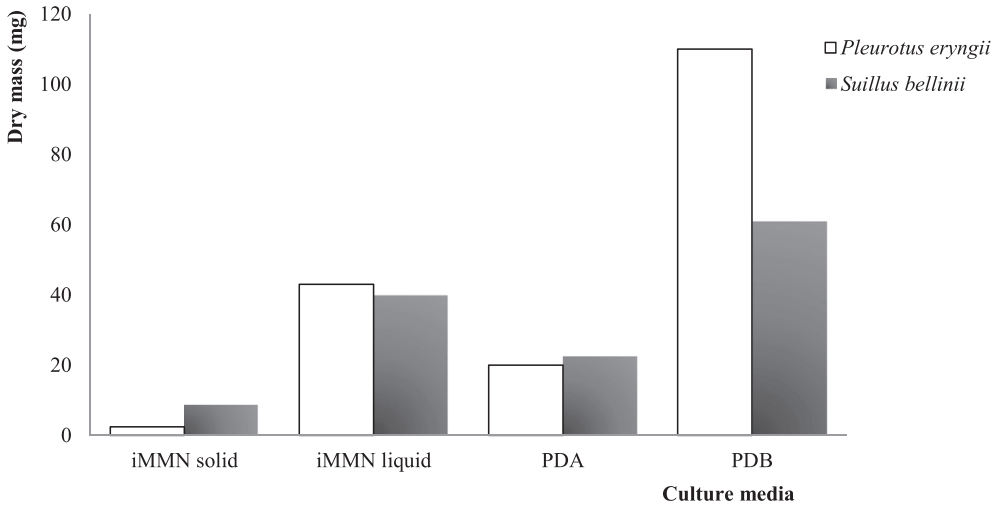


Fig. 2. Average biomass of *P. eryngii* and *S. bellinii* mycelia (mg/Petri dish or Flask).

Table 1
Ergosterol content (mg/g extract) and phenolic acids composition (μg/ g extract) in the mycelia and culture media of *P. eryngii* and *S. bellinii*. The values corresponding to the fruiting body of both mushrooms (wild samples) are also presented. Values are given as mean ± standard deviation.

		Ergosterol	Protocatechuic acid	*p*-Hydroxybenzoic acid	*p*-Coumaric acid	Cinnamic acid
Pleurotus eryngii						
Fruiting body (wild)		4.1 ± 0.3	nd	273 ± 10	42 ± 4	61 ± 4
iMMN liquid	Mycelium	0.6 ± 0.1^{b}	nd	186 ± 8^{a}	nd	34 ± 3^{b}
	Culture medium	nd	nd	nd	nd	nd
PDB	Mycelium	0.10 ± 0.02^{c}	nd	122 ± 6^{c}	nd	49 ± 4^{a}
	Culture medium	nd	nd	nd	nd	nd
iMMN solid	Mycelium	0.9 ± 0.1^{a}	nd	149 ± 9^{b}	nd	15 ± 2^{c}
PDA	Mycelium	1.0 ± 0.1^{a}	nd	178 ± 8^{a}	nd	15 ± 2^{c}
p-values (n = 54)	Homoscedasticity[1]	<0.001	–	<0.001	–	<0.001
	1-way ANOVA[2]	<0.001	–	<0.001	–	<0.001
Suillus bellinii						
Fruiting body (wild)		6.5 ± 0.4	403 ± 16	1821 ± 97	nd	39 ± 4
iMMN Liquid	Mycelium	8.9 ± 0.5^{b}	nd	213 ± 7^{c}	nd	130 ± 1^{a}
	Culture medium	nd	nd	nd	nd	nd
PDB	Mycelium	12.4 ± 0.5^{a}	nd	204 ± 11^{c}	nd	111 ± 4^{b}
	Culture medium	nd	nd	nd	nd	nd
iMMN solid	Mycelium	9.0 ± 0.4^{b}	nd	394 ± 15^{a}	nd	25 ± 2^{d}
PDA	Mycelium	3.5 ± 0.3^{c}	nd	372 ± 16^{b}	nd	36 ± 3^{c}
p-value (n = 54)	Homoscedasticity[1]	<0.001	–	<0.001	–	<0.001
	1-way ANOVA[2]	<0.001	–	<0.001	–	<0.001

[1] Homoscedasticity among culture components was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$.
[2] $p < 0.05$ indicates that the mean value of at least one component differs from the others (in this case, multiple comparison tests were performed). For each culture component, means within a column with different letters differ significantly ($p < 0.05$).

(Barreira et al., 2014), but that higher value was measured in commercial samples, which might justify the difference in comparison to the result reported here.

Among the phenolic acids, *p*-hydroxybenzoic acid was the major compound in both mushrooms, reaching quantities nearly sevenfold higher in *S. bellinii* (1821 μg/g extract). Nevertheless, the main phenolic acid in *S. bellinii* was previously reported as being *p*-hydroxyphenylacetic acid (45 μg/100 g fresh weight), among *p*-hydroxybenzoic acid derivatives, and *o*-coumaric acid (15 μg/100 g fresh weight), in what concerns hydroxycinnamic acids. In the same study, the concentration of *p*-hydroxybenzoic was 7 μg/100 g fresh weight (Kalogeropoulos et al., 2013), which is in the range of the concentrations detected herein, considering typical moisture contents (≈90%) and extraction yields (≈5%) for this particular mushroom species.

Concerning *P. eryngii*, the phenolic acids profile is in agreement with previous works conducted with samples from the same geographic area, but different harvesting years (Ferreira et al., 2009; Heleno, Martins, Queiroz, & Ferreira, 2015; Reis et al., 2014). Even so, in a similar study, syringic acid and vanillic acid were reported in quantities similar to *p*-hydroxybenzoic acid (Lin et al., 2014).

3.2. Antioxidant activity

The results obtained for each of the performed antioxidant activity assays, given as EC_{50} values, are shown in Table 2. In all evaluated cases, the highest activity was obtained on the TBARS formation inhibition assay (*P. eryngii*: 0.11 mg/mL extract; *S. bellinii*: 0.011 mg/mL extract), followed by β-carotene bleaching inhibition (*P. eryngii*: 0.45 mg/mL extract; *S. bellinii*: 0.12 mg/mL extract), reducing power (*P. eryngii*: 0.98 mg/mL extract; *S. bellinii*: 0.16 mg/mL extract) and scavenging effects on DPPH radicals (*P. eryngii*: 12.8 mg/mL extract; *S. bellinii*: 0.61 mg/mL extract). In line with its higher quantities of phenolic acids, the fruiting bodies of *S. bellinii* showed higher antioxidant activity than *P. eryngii*, sometimes 10-fold or 20-fold higher, such as verified in the TBARS

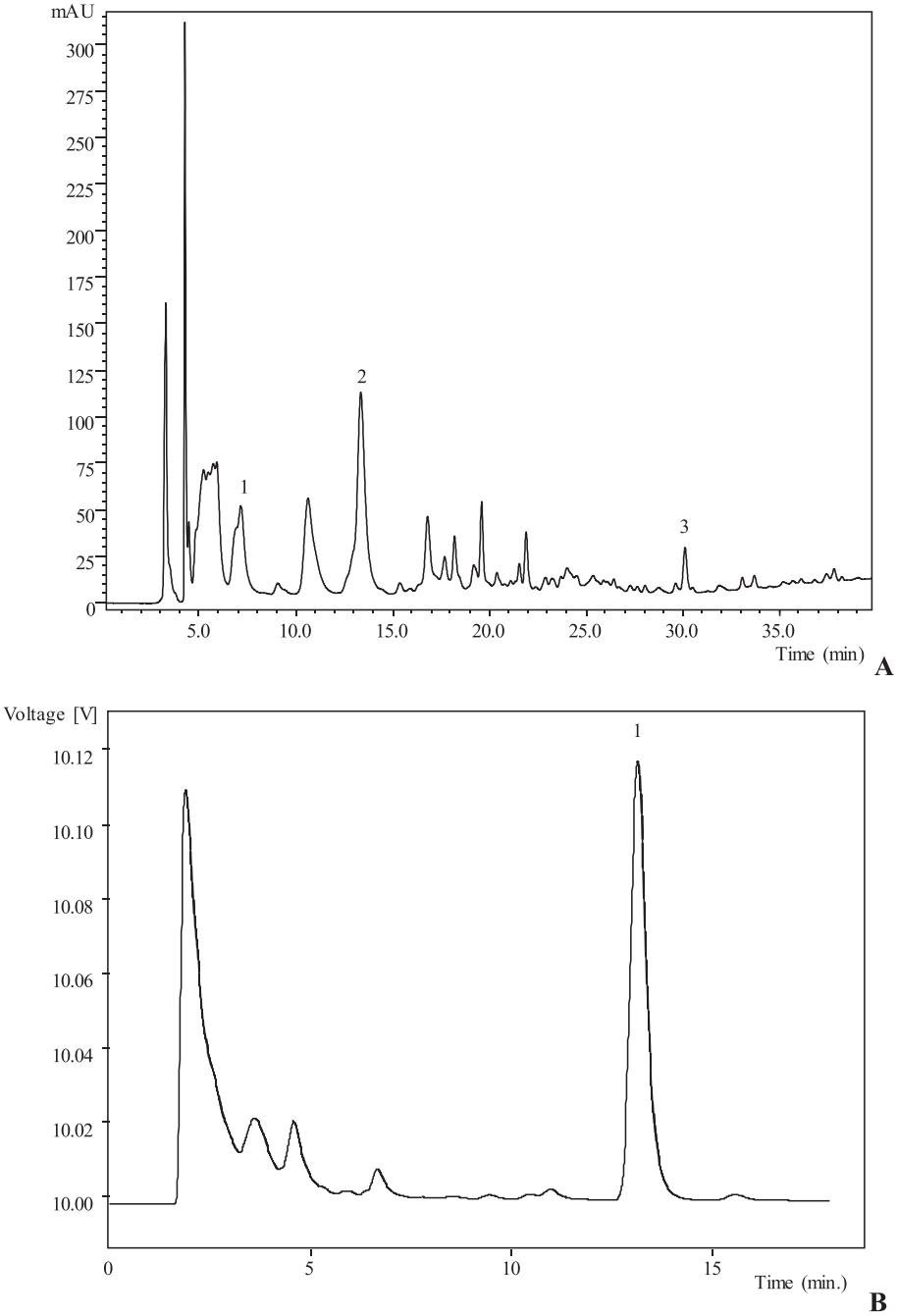


Fig. 3. Individual profile of (A) phenolic compounds present in *S. bellinii* fruiting body recorded at 280 nm (1- protocatechuic acid; 2- *p*-hydroxybenzoic acid; 3- cinnamic acid) and (B) ergosterol present in *S. bellinii* mycelium grown on PDB (1- ergosterol).

formation inhibition and DPPH scavenging activity, respectively. Nevertheless, the values obtained for *P. eryngii* represent higher antioxidant activity (except for the DPPH scavenging activity assay) than that reported previously (Lin et al., 2014; Reis et al., 2012).

Regarding *S. bellinii*, an activity of 35% in DPPH scavenging activity, at 0.15 mg/mL was previously reported (Ribeiro et al., 2006), which is slightly better than the 50% activity obtained in this study for the 0.61 mg/mL concentration. In a similar study, performed with Greek samples of *S. bellinii*, this mushroom also showed high radical scavenging activity and reducing power, but the results are not directly comparable, because they were given in trolox equivalents (Kalogeropoulos et al., 2013).

Considering the main purpose of this work, it was very interesting to discover that the antioxidant activity measured in the mycelia and in the culture media was very close (in some cases better) to that verified in the fruiting bodies, emphasizing the high potential of these fungal culture components. In the case of the culture media, these results have an increased interest, since those components are usually considered as by-products of mushroom cultivation. Furthermore, the differences among the same culture media, after having been used to grow each of the mushrooms, indicate that the measured antioxidant activity is in fact due to the mycelium, and not to the culture media components. Furthermore, some compounds responsible for the antioxidant activity are not

Table 2
Antioxidant activity (EC_{50} values, mg/mL extract) of the mycelia and culture media of *P. eryngii* and *S. bellinii*. The values corresponding to the fruiting body of both mushrooms (wild samples) are also presented. Values are given as mean ± standard deviation.

		DPPH scavenging activity	Reducing power	β-carotene bleaching inhibition	TBARS formation inhibition
Pleurotus eryngii					
Fruiting body (wild)		12.8 ± 0.2	0.98 ± 0.01	0.45 ± 0.02	0.11 ± 0.01
iMMN liquid	Mycelium	24.9 ± 0.3^{b}	1.35 ± 0.02^{a}	0.44 ± 0.03^{c}	0.17 ± 0.01^{b}
	Culture medium	28.2 ± 0.5^{a}	1.24 ± 0.05^{b}	0.57 ± 0.01^{a}	0.20 ± 0.02^{a}
PDB	Mycelium	19.7 ± 0.5^{d}	1.10 ± 0.01^{c}	0.40 ± 0.05^{c}	0.15 ± 0.01^{c}
	Culture medium	22.0 ± 0.5^{c}	0.95 ± 0.02^{e}	0.52 ± 0.02^{b}	0.17 ± 0.01^{b}
iMMN solid	Mycelium	17.1 ± 0.5^{e}	1.08 ± 0.01^{c}	0.23 ± 0.01^{d}	0.13 ± 0.01^{d}
PDA	Mycelium	16.1 ± 0.5^{f}	1.03 ± 0.01^{d}	0.18 ± 0.02^{e}	0.11 ± 0.01^{e}
p-value (n = 54)	Homoscedasticity[1]	0.015	<0.001	<0.001	<0.001
	1-way ANOVA[2]	<0.001	<0.001	<0.001	<0.001
Suillus bellinii					
Fruiting body (wild)		0.61 ± 0.01	0.16 ± 0.01	0.12 ± 0.01	0.011 ± 0.001
iMMN liquid	Mycelium	1.10 ± 0.01^{b}	0.84 ± 0.02^{b}	0.21 ± 0.03^{b}	0.14 ± 0.01^{a}
	Culture medium	1.18 ± 0.02^{a}	0.93 ± 0.02^{a}	0.29 ± 0.01^{a}	0.15 ± 0.01^{a}
PDB	Mycelium	0.83 ± 0.02^{d}	0.24 ± 0.01^{d}	0.17 ± 0.01^{d}	0.010 ± 0.001^{c}
	Culture medium	0.95 ± 0.03^{c}	0.29 ± 0.02^{c}	0.20 ± 0.01^{c}	0.014 ± 0.002^{bc}
iMMN solid	Mycelium	0.62 ± 0.03^{e}	0.17 ± 0.01^{e}	0.14 ± 0.02^{e}	0.016 ± 0.001^{b}
PDA	Mycelium	0.59 ± 0.02^{f}	0.16 ± 0.01^{e}	0.14 ± 0.01^{e}	0.011 ± 0.001^{c}
p-value (n = 54)	Homoscedasticity[1]	0.003	<0.001	<0.001	<0.001
	1-way ANOVA[2]	<0.001	<0.001	<0.001	<0.001

[1] Homoscedasticity among culture components was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$.
[2] $p < 0.05$ indicates that the mean value of at least one component differs from the others (in this case, multiple comparison tests were performed). For each culture component, means within a column with different letters differ significantly ($p < 0.05$).

released to the culture medium; they are kept in the mycelium. Without exception, the mycelia and the culture media of *S. bellinii* gave better results than the same components of *P. eryngii*.

In coherence with the observed for the fruiting bodies, the results obtained with *P. eryngii* mycelia were better than the reported previously, except for DPPH scavenging activity (Reis et al., 2012).

In general, the detected antioxidant activity represents an important added-value, despite the weaker performance of the mycelium and culture medium extracts when compared with the DPPH scavenging activity (EC_{50} values ranging from 0.8 to 3 mg/mL) and reducing power (EC_{50} = 0.5 mg/mL) obtained in polysaccharide fractions of *P. eryngii* (Li & Shah, 2014; Ma et al., 2016; Zhang et al., 2016). However, it should be reminded that the results presented herein were obtained with raw extracts, not with purified fractions.

Due to the lack of studies with the mycelia of *S. bellinii*, no comparisons could be performed.

3.3. Anti-inflammatory activity

Macrophages, the main components of the innate immune system, have essential regulation functions in several immunopathological conditions during the inflammatory process (Yoon et al., 2009; Yuan, Wahlqvist, He, Yang, & Li, 2006). However, the overproduction of inflammatory mediators in the course of uncontrolled inflammation processes might cause adverse consequences in the pathogenesis of many inflammatory diseases such as cancer, diabetes and cardiovascular disease (Yuan et al., 2006). Endotoxin lipopolysaccharide (LPS) is able to induce the production of mediators like nitric oxide (NO), pro-inflammatory cytokines (besides inhibiting anti-inflammatory cytokines) and tumor necrosis factor in macrophages. Thereby, macrophages stimulated by LPS have been widely used for anti-inflammatory activities evaluation *in vitro* (García-Lafuente, Guillamón, Villares, Rostagno, & Martínez, 2009). In addition, due to the reproducible response of RAW264.7 macrophages to LPS, this cell line has been widely used for inflammatory research (Huang & Ho, 2010).

Accordingly, the anti-inflammatory activity was evaluated using a LPS-stimulated RAW264.7 cell line (Table 3). The highest activity was measured in the methanolic extracts prepared from the fruiting bodies of *S. bellinii* (EC_{50} = 90 μg/mL extract). The extracts of *P. eryngii* were also able to suppress NO production, but in less extent (EC_{50} = 223 μg/mL extract). However, these results were better than those obtained in previous assays (Taofiq et al., 2015).

Regarding the evaluated culture components, some interesting results were obtained, especially for the mycelia of *P. eryngii* grown in solid media, which showed higher anti-inflammatory activity (EC_{50} = 184–189 μg/mL extract) than the corresponding fruiting bodies, highlighting their possible use in anti-inflammatory applications.

Moro et al. (2012) found that the phenolic compounds of the fruiting bodies might contribute to their anti-inflammatory activities, inducing inhibition of NO production and iNOS expression in LPS-activated RAW264.7 cells. However, the anti-inflammatory activity of the mycelia of *S. bellinii* was not maintained in neither of the culture components, indicating that the anti-inflammatory effects of the extracts appeared to be related with other components besides ergosterol and phenolic acids. Considering the chemical composition of mushroom, this activity might be related with compounds such as heteropolysaccharides (Lima et al., 2016), triterpenes (Choi et al., 2014) or carotenoids (Moro et al., 2012).

3.4. Cytotoxicity

The results for the anti-proliferative activity assayed in four human tumor cell lines (MCF-7, NCI-H460, HeLa and HepG2) and a porcine liver primary cell line (PLP2) are shown in Table 3.

The extracts prepared from the fruiting bodies of wild *S. bellinii* samples showed higher activity against MCF7 (GI_{50} = 70 μg/mL extract), NCI-H460 (GI_{50} = 65 μg/mL extract) and HepG2 (GI_{50} = 68 μg/mL extract). However, the same extracts could not inhibit the HeLa cell line (up to the maximum assayed concentration: 400 μg/mL extract). On the other hand, the extracts from the fruiting bodies of wild *P. eryngii* samples had a similar behavior in all cell lines (GI_{50} values varying from 224 μg/mL extract in HepG2 to 246 μg/mL extract in MCF7). The anti-proliferative activity of *P. eryngii* extracts (specifically, its polysaccharides fraction) was previously reported in HepG2, where it induced apoptosis, cell cycle

Table 3
Anti-proliferative (GI_{50} values, μg/mL extract) extract and anti-inflammatory activity (EC_{50} values, μg/mL extract) of the mycelia and culture media of *P. eryngii* and *S. bellinii*. The values corresponding to the fruiting body of both mushrooms (wild samples) are also presented. Values are given as mean ± standard deviation.

		MCF-7	NCI-H460	HeLa	HepG2	PLP2	RAW264.7
Pleurotus eryngii							
Fruiting body (wild)		246 ± 6	237 ± 10	243 ± 10	224 ± 12	>400	223 ± 9
iMMN liquid	Mycelium	242 ± 7^{c}	275 ± 12 c^{d}	265 ± 4^{d}	280 ± 3^{c}	>400	>400^{a}
	Culture medium	>400^{a}	>400^{a}	>400^{a}	>400^{a}	>400	>400^{a}
PDB	Mycelium	270 ± 15^{b}	268 ± 5^{d}	280 ± 1^{c}	224 ± 11^{d}	>400	308 ± 10^{b}
	Culture medium	>400^{a}	>400^{a}	>400^{a}	>400^{a}	>400	>400^{a}
iMMN solid	Mycelium	169 ± 12^{d}	363 ± 13^{b}	302 ± 9^{b}	314 ± 2^{b}	>400	189 ± 7^{c}
PDA	Mycelium	173 ± 8^{d}	285 ± 10^{c}	267 ± 20^{d}	140 ± 6^{e}	>400	184 ± 6^{c}
p-value (n = 54)	Homoscedasticity	<0.001	<0.001	<0.001	<0.001	–	<0.001
	1-way ANOVA	<0.001	<0.001	<0.001	<0.001	–	<0.001
Suillus bellinii							
Fruiting body (wild)		70 ± 2	65 ± 3	>400	68 ± 3	215 ± 38	90 ± 2
iMMN liquid	Mycelium	>400^{a}	>400^{a}	>400	>400^{a}	>400	>400
	Culture medium	>400^{a}	>400^{a}	>400	>400^{a}	>400	>400
PDB	Mycelium	300 ± 7^{b}	279 ± 2^{b}	>400	226 ± 5^{b}	>400	>400
	Culture medium	>400^{a}	>400^{a}	>400	>400^{a}	>400	>400
iMMN solid	Mycelium	>400^{a}	>400^{a}	>400	>400^{a}	>400	>400
PDA	Mycelium	289 ± 11^{c}	257 ± 2^{c}	>400	206 ± 11^{c}	>400	>400
p-value (n = 54)	Homoscedasticity	<0.001	<0.001	–	<0.001	–	–
	1-way ANOVA	<0.001	<0.001	–	<0.001	–	–

[1]Homoscedasticity among culture components was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$.
[2]$p < 0.05$ indicates that the mean value of at least one component differs from the others (in this case, multiple comparison tests were performed). For each culture component, means within a column with different letters differ significantly ($p < 0.05$).

arrest at the S-phase and intracellular production of reactive oxygen species (Yang et al., 2013). Besides its *in vitro* anti-tumoral activity, *P. eryngii* was previously reported as being active against mice renal cancer *in vivo* (Yang et al., 2013). Despite their generally lower activity against tumor cell lines, the extracts from *P. eryngii* did not exhibit a toxic effect on the primary cell line, contrarily with the observed for *S. bellinii* fruiting bodies.

Interestingly, none of the assayed culture components inhibit the growth of PLP2 cell line, which constitutes a good indicator of the lack of toxicity of the mycelia and culture media of both mushroom species in non-tumor cell lines.

Concerning the tumor cell lines, the mycelia of *P. eryngii* showed similar cytotoxicity to the fruiting bodies. In fact, the measured activity was often higher than the observed for the fruiting bodies. In some cases, such as the mycelium grown in PDA medium, the GI_{50} values are comparable to those obtained with purified polysaccharide fractions (Ma et al., 2014, 2016; Ren et al., 2016), which represents a very interesting result.

On the other hand, the mycelia of *S. bellinii* showed significantly lower anti-proliferative activity, when compared to the fruiting bodies.

The culture media did not show any anti-proliferative activity (up to the maximum assayed concentrations) in both mushroom species

4. Conclusion

This study was initially designed to evaluate the mycelia of *P. eryngii* and *S. bellinii*, as well as their culture media, as potential alternative sources of bioactive compounds or as ingredients to be used in applications with antioxidant, anti-inflammatory or cytotoxic activities. In general, *S. bellinii* mycelia showed higher contents of ergosterol and phenolic compounds, which were also more abundant in the fruiting body of this species. Likewise, the antioxidant activity was also higher among the *S. bellinii* components. However, these extracts did not show anti-inflammatory activity up to the maximum assayed concentrations, contrarily to the observed for the mycelia of *P. eryngii*. Furthermore, the latter component showed a cytotoxicity similar (and often superior) to its fruiting bodies, in opposition to *S. bellinii*, whose mycelia showed a significant loss of anti-proliferative activity. In general, each culture component showed differentiated activity, which should be considered together with the growth rate and biomass yielded for each mushroom.

Acknowledgments

The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) and FEDER under Programe PT2020 for financial support to CIMO (UID/AGR/00690/2013), LSRE (Project UID/EQU/50020/2013), J.C.M. Barreira (BPD/72802/2010), L. Barros (SFRH/BPD/107855/2015), and R.C. Calhelha (SFRH/BPD/BPD/68344/2010) grants.

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