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Bioactive formulations prepared from fruiting bodies and submerged culture mycelia of the Brazilian edible mushroom *Pleurotus ostreatoroseus* Singer

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Pleurotus ostreatoroseus is a Brazilian edible mushroom whose chemical characterization and bioactivity still remain underexplored. In this study, the hydrophilic and lipophilic compounds as well as the antioxidant, anti-inflammatory and antimicrobial activities of formulations (ethanol extracts) prepared with its fruiting bodies and submerged culture mycelia were compared. The bioactive formulations contain at least five free sugars, four organic acids, four phenolic compounds and two tocopherols. The fruiting body-based formulation revealed higher reducing power, DPPH scavenging activity, β-carotene bleaching inhibition and lipid peroxidation inhibition in brain homogenates than the mycelium-based preparation, as well as higher anti-inflammatory and antimicrobial activities. The absence of hepatotoxicity was confirmed in porcine liver primary cells. These functional responses can be related to the levels of bioactive components including phenolic acids, organic acids and tocopherols.

Introduction

Besides their worldwide-appreciated flavour, mushrooms offer exceptional nutritional value, with low lipid content and large amounts of carbohydrates and proteins, in addition to essential amino acids and fat-soluble vitamins (vitamins A, D, E and K).[1] Moreover, mushrooms are increasingly attractive as functional foods and as potential sources for the development of new drugs.[2]

Among the bioactive compounds found in mushrooms, tocopherols,[3] phenolic compounds[4] and some organic acids[5] have been implicated for their nutraceutical potential[6,7] and bioactivity, including their antioxidant[8,9] and antimicrobial[10] effects.

Pleurotus is an important genus of basidiomycetes, especially; those occurring in the subtropics and tropics, which occupy the third position in the production of edible mushrooms.[11,12] *Pleurotus* spp. can be easily cultivated due to their ability to colonise and degrade a wide variety of substrates containing cellulose, hemicellulose and lignin, using them in their own development.[12,13] Furthermore, these species have a quick mycelium growth and fruiting, and a low cost of culture.[14,15] For these reasons, as well as their well-known nutritional and functional characteristics, *Pleurotus* spp. have become very interesting from a commercial point of view.[12]

Pleurotus ostreatoroseus Singer is an edible Brazilian mushroom that stands out for its characteristic rosy coloration and the delightful flavour of its fruiting bodies. It was firstly described by Singer[16] from sample material collected at *Dois Irmãos* Park (Brazilian Atlantic Forest), Recife, PE, Brazil. This species is included among the white rot fungi for its excellent potential in lignin degradation and is considered an autochthonous mushroom in the tropics; it grows quite well in tropical temperatures.[17]

Although a few studies have covered the chemical characterization and antitumor and immunomodulatory effects of polysaccharides isolated from *P. ostreatoroseus* fruiting bodies,[18–20] the chemical elucidation and bioactivity of other molecules, such as phenolic compounds, still remain unknown.

Regarding antioxidant, anti-inflammatory and antimicrobial effects of *Pleurotus* spp., there are some studies with *Pleurotus ostreatus* (Jacq. ex Fr.) P. Kumm, *Pleurotus cystidiosus* O.K. Mill, *Pleurotus pulmonarius* (Fr.) Quél, *Pleurotus djamor*

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(Rumph. ex Fr.), *Pleurotus citrinopileatus* Singer and *Pleurotus eryngii* (DC.) Quél.[9,10,21–23] Nevertheless, to the authors' knowledge, there are no previous reports on antioxidant, anti-inflammatory and antimicrobial activities of *P. ostreatoroseus*.

In the present work, a study was performed with the fruiting body and mycelium of *P. ostreatoroseus*, by preparing bioactive formulations (ethanolic extracts) that were further characterized in terms of hydrophilic and lipophilic compounds. The antioxidant, anti-inflammatory and antimicrobial potential of the prepared extracts were evaluated and compared, and non-toxicity was confirmed in a primary cell culture of porcine liver cells.

Experimental

Fruiting body selection and nutritional characterization

Fruiting bodies (basidiocarps) of *P. ostreatoroseus* were obtained from a local producer in Maringá, PR, Brazil, in spring 2014. The fruiting bodies were selected in accordance with the commercial requirements in Brazil, *i.e.*, before the rupture of the veil (closed cap), in order to preserve the sensory characteristics as well as firmness, which also reduces fragmentation during processing.

The fruiting bodies were nutritionally characterized regarding moisture, proteins, fat, carbohydrates and ash, by using the standard procedures.[24] The crude protein content (N × 4.38) was estimated by the macro-Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600 ± 15 °C. Total fibre was determined by enzymatic–gravimetric method.[24] Carbohydrate value was calculated by difference.

Production of the *P. ostreatoroseus* mycelia

A commercial isolate of *P. ostreatoroseus* was obtained from a local producer. The stock culture was maintained on wheat bran extract agar slants (Fig. 1) and sub-cultured every month. The slants were incubated at 28 °C for 7 days and then stored at 4 °C in a refrigerator for up 30 days. The inocula were prepared by adding actively growing mycelia from a newly prepared slant culture (5 mycelial agar discs with 0.5 cm diameter) into 50 mL medium in a 250 mL Erlenmeyer flask. They were incubated for 5 days at 28 °C on a rotary shaker at 160 rpm. The wheat bran extract medium was prepared with 100 g of wheat bran that was boiled in 1 L of distilled water. The mixture was then filtered in gauze, and mineral solution[25] at a final concentration of 2% was added to the filtrate. For the submerged culture, 150 mL of the same medium was prepared in a 500 mL flask, and pre-culture broth was inoculated (at 1.0 mL L^{-1}). The flasks were incubated at 28 °C on a rotary shaker at 160 rpm for up to 7 days. The mycelia were recovered from the liquid medium by filtration, washed with distilled water, immediately stored in the freezer and posteriorly freeze-dried.

Preparation of the bioactive formulations

The extraction procedure followed the methodology proposed by Carvajal *et al.*,[26] with ethanol chosen as the extractor solvent due to its low cost, abundance and lower toxicity in comparison with other organic solvents. Fruiting bodies were dried and milled to a fine powder (40 mesh) while the previously freeze-dried mycelia were milled to the same granulometry. The samples (5 g) were extracted by stirring with 100 mL of ethanol 70 : 30 (in water) at 25 °C and at 130 rpm for 3 h and filtered through Whatman no 1 paper. The extraction procedure was repeated twice. The combined filtrates were concentrated with a rotary vacuum evaporator at 40 °C in order to eliminate the solvent and posteriorly freeze-dry the formulation. The freeze-dried powders were stored in the freezer until use. The extraction yield was about 20% for both basidioma and mycelium samples.

Standards and reagents

Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), sugar, organic acid and phenolic compound standards were from Sigma (St. Louis, MO, USA). Racemic tocol (50 mg mL^{-1}) and tocopherols, were purchased from Matreya (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Dimethylsulfoxide (DMSO)

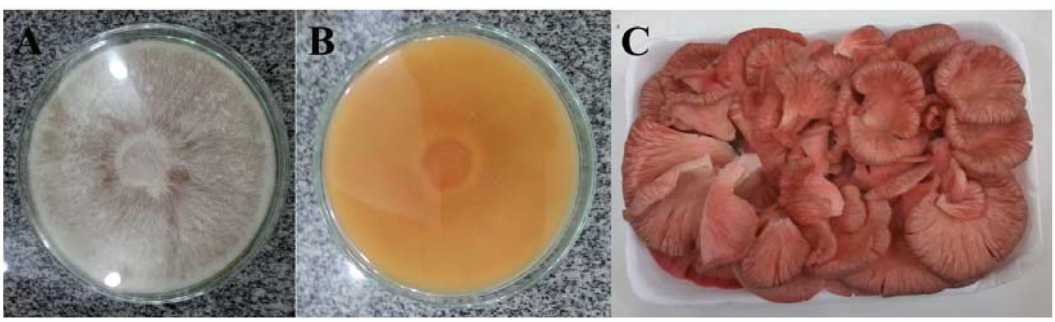

Fig. 1 Morphological characteristics of *P. ostreatoroseus*. (a) Mycelium cultivated on wheat bran extract agar slants. (b) Bottom of the mycelium plate of figure a in which the characteristic rosy coloration can be observed. (c) Fruiting bodies of commercial package.

(Merck KGaA, Germany) was used as a solvent in antimicrobial assays. Dulbecco's modified Eagle's medium, Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA, penicillin/streptomycin solution (100 U mL^{-1} and 100 μg mL^{-1}, respectively) were purchased from Gibco Invitrogen Life Technologies (California, USA). Sulforhodamine B, trypan blue, trichloroacetic acid (TCA) and Tris were purchased from Sigma Chemical Co. (Saint Louis, USA). RAW264.7 cells were purchased from ECACC ("European Collection of Animal Cell Culture") (Salisbury, UK), lipopolysaccharide (LPS) from Sigma and DMEM medium from HyClone. The Griess Reagent System Kit was purchased from Promega, and dexamethasone from Sigma. Ethanol and all other chemicals were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

Chemical characterization of the *P. ostreatoroseus* formulations

Free sugars. The extracts (500 mg) were spiked with the Internal Standard, IS (raffinose, 5 mg mL^{-1}), re-dissolved in water (5 mL) and successively defatted three times with 10 mL of ethyl ether. After ethyl ether removal, the residues were filtered through a 0.22 μm disposable LC filter disk and transferred into an injection vial. Analysis was performed by high performance liquid chromatography (HPLC). The total system consisted of an integrated system with a pump (Knauer, Smartline system1000, Berlin, Germany), degasser system (Smartline manager 5000) and an auto-sampler (AS-2057 Jasco, Easton, MD, USA), coupled to a refraction index detector (RI detector Knauer Smartline 2300), as previously described by the authors.[27] The chromatographic separation was achieved with an Eurospher 100-5 NH_2 column (4.6 mm × 250 mm, 5 μm, Knauer) operating at 35 °C (7971R Grace oven). The mobile phase used was acetonitrile/deionized water, 70 : 30 (v/v) at a flow rate of 1 mL min^{-1}. Sugar identification was made by comparing the relative retention times of sample peaks with standards. Data were analyzed using Clarity 2.4 Software (DataApex). Quantification was based on the RI signal response of each standard, using the internal standard (IS, raffinose) method and by using calibration curves obtained from the commercial standards of each compound. The results were expressed in mg per g of extract.

Organic acids. The extracts (50 mg) were re-dissolved in *meta*-phosphoric acid (2 mL) and subsequently filtered through Whatman no 4 paper. Organic acids were determined by ultra-fast liquid chromatography (UFLC, Shimadzu 20A series, Shimadzu Corporation, Kyoto, Japan) coupled with a photodiode array detector (PDA) as previously described by the authors.[5] Separation was achieved on a Sphere Clone (Phenomenex) reverse phase C_{18} column (5 μm, 250 mm × 4.6 mm i.d) thermostatted at 35 °C. The elution was performed with 3.6 mM sulphuric acid using a flow rate of 0.8 mL min^{-1}. The organic acids were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in mg per g of extract.

Phenolic acids. The extracts were re-dissolved in ethanol : water (20 : 80, v/v) and filtered through a 0.22 μm disposable LC filter disk for HPLC analysis. Phenolic acid determination was performed using the UFLC mentioned above, as previously described by Reis *et al.*[28] Separation was achieved with a Waters Spherisorb S3 ODS-2 C_{18}, 3 μm (4.6 mm × 150 mm) column, thermostatted at 35 °C. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic at 15% B for 5 min, 15% B to 20% B over 5 min, 20–25% B over 10 min, 25–35% B over 10 min, 35–50% B for 10 min, and then re-equilibration of the column, using a flow rate of 0.5 mL min^{-1}. Double online detection was carried out in the DAD using 280 nm as the preferred wavelength and in a mass spectrometer (MS) connected to a HPLC system *via* the DAD cell outlet. The phenolic compounds were characterized according to the UV and mass spectra, retention times, and comparison with authentic standards. The identified phenolic acids were quantified by comparison of the area of their peaks recorded at 280 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in μg per g of extract.

Tocopherols. BHT solution (10 mg mL^{-1}, 100 μL) and IS solution (tocol 2 μg mL^{-1}, 250 μL) were added to the extracts (described above) prior to the extraction procedure. The extracts were homogenized with methanol (4 mL) by vortex mixing (1 min). Subsequently, hexane (4 mL) was added and the extracts were again vortex mixed for 1 min. After that, a saturated NaCl aqueous solution (2 mL) was added, the mixture was homogenized (1 min), centrifuged (5 min, 4000*g*) and the clear upper layer was carefully transferred to a vial. The sample was re-extracted twice with hexane. The combined extracts were taken to dryness under a nitrogen stream, re-dissolved in 1 mL of hexane, dehydrated with anhydrous sodium sulfate, filtered through a 0.22 μm disposable LC filter disk, and transferred into a dark injection vial. Analysis was performed by HPLC (equipment described above), and a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm, as previously described by the authors.[27] The chromatographic separation was achieved with a Polyamide II (250 × 4.6 mm) normal-phase column from YMC Waters operating at 35 °C. The mobile phase used was a mixture of hexane and ethyl acetate (70 : 30, v/v) at a flow rate of 1 ml min^{-1}. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in μg per g of extract.

Bioactivity of the *P. ostreatoroseus* formulations

Antioxidant activity. Successive dilutions of the stock solution were made and used for *in vitro* assays as described by Reis *et al.*,[28] to evaluate the antioxidant activity of the samples.

The sample concentrations (mg mL^{-1}) 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 (ferricyanide/Prussian blue assay) against the sample concentrations. Trolox was used as a positive control.

Ferricyanide/prussian blue assay. The extract solutions with different concentrations (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). Each 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 into the 48 wells plate in addition to deionized water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm in an ELX800 Microplate Reader (Bio-Tek Instruments, Inc.; Winooski, VT, USA).

DPPH radical-scavenging activity assay. This methodology was performed using the Microplate Reader mentioned above. The reaction mixtures in the 96 well plate consisted of extract solutions of different concentrations (30 μL) and a methanolic solution (270 μL) containing DPPH radicals (6×10^{-5} mol L^{-1}). Each mixture was left to stand for 30 min in the dark, and the absorption was measured at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of the DPPH discoloration using the equation: $\%RSA = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution.

Inhibition of β-carotene bleaching or β-carotene/linoleate assay. A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two milliliters of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40 °C under vacuum and 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 test tubes containing extract solutions at different concentrations (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.

Thiobarbituric acid reactive substances (TBARS) assay. Porcine (*Sus scrofa*) brains were obtained from officially slaughtered animals, dissected, and homogenized with Polytron in an 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 concentration sample 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 (%) = $[(A - B)/A] \times 100\%$, where *A* and *B* are the absorbances of the control and the sample solution, respectively.

Anti-inflammatory activity. The extracts were dissolved in water, initially concentrated at 8 mg mL^{-1} and then further dilutions were prepared from 8 mg mL^{-1} to 0.125 mg mL^{-1}. The mouse macrophage-like cell line RAW264.7 was cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum and glutamine at 37 °C under 5% CO_2, in humidified air. For each experiment, cells were detached with a cell scraper. Under our experimental cell density (5×10^5 cells per mL), the proportion of dead cells was less than 1%, according to Trypan blue dye exclusion tests. Cells were seeded in 96-well plates at 150 000 cells per well and allowed to attach to the plate overnight. Then, cells were treated with the different concentrations of each of the extracts for 1 h. Dexamethasone (50 μM) was used as a positive control for the experiment. The following step was stimulation with LPS (1 μg mL^{-1}) for 18 h. The effect of the tested samples in the absence of LPS was also evaluated, in order to observe if they induced changes in NO basal levels. In negative controls, no LPS was added. Both extracts and LPS were dissolved in supplemented DMEM. For the determination of nitric oxide, a Griess Reagent System kit (Promega) was used, which contains sulfanilamide, NED and nitrite solutions. One hundred microliters of each cell culture supernatant were transferred to the plate in duplicate and mixed with sulfanilamide and NED solutions, for 5–10 minutes each, at room temperature. The nitrite produced was determined by measuring the optical density at 515 nm, in the microplate reader referred to above, and was compared to the standard calibration curve.

Antibacterial activity. The following Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030), and Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973) were used. The microorganisms were obtained from the Mycological laboratory, Department of Plant Physiology, Institute for biological research "Siniša Stanković", University of Belgrade, Serbia.

The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined by the microdilution method.[29] Each fresh overnight culture of bacteria was adjusted spectrophotometrically to a concentration of 1×10^5 CFU mL^{-1}. The requested CFU mL^{-1} corresponded to a bacterial suspension determined in a spectrophotometer at 625 nm (OD625). Dilutions of inocula were cultured on solid medium to verify the absence of contamination and check the validity of each inoculum. Different solvent dilutions of the ethanolic extract were added to the wells containing 100 μL of Tryptic Soy Broth (TSB) and afterwards, 10 μL of inoculum was added to all wells. The microplates were incubated for 24 h at 37 °C. The MIC of the samples was detected following the addition of 40 μL of iodonitrotetrazolium chloride (INT)

(0.2 mg mL^{-1}) and incubation at 37 °C for 30 min. The lowest concentration that produced a significant inhibition (around 50%) of the growth of the bacteria in comparison with the positive control was identified as the MIC. The minimum inhibitory concentrations (MICs) obtained from the susceptibility testing of various bacteria to tested extracts were determined also by a colorimetric microbial viability assay based on the reduction of the INT color and compared with a positive control for each bacterial strain.[30] MBC was determined by serial sub-cultivation of 10 μL into microplates containing 100 μL of TSB. The lowest concentration that showed no growth after this sub-culturing was read as the MBC. Standard drugs, namely streptomycin and ampicillin, were used as positive controls. 5% DMSO was used as the negative control. Samples were tested in duplicate and experiments were repeated three times.

Antifungal activity. For the antifungal bioassays, the following microfungi were used: *Aspergillus fumigatus* (ATCC 1022), *Aspergillus versicolor* (ATCC 11730), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112), and *Penicillium verrucosum* var. *cyclopium* (food isolate). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. The micromycetes were maintained on malt agar (MA) and the cultures were stored at 4 °C and sub-cultured once a month.[31]

The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 μL per well. The inocula were stored at 4 °C for further use. Dilutions of each inoculum were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculum.

Minimum inhibitory concentration (MIC) determination was performed by a serial dilution technique using 96-well microtitre plates. The investigated extract was dissolved in a 5% solution of DMSO and added to broth malt medium with a fungal inoculum. The microplates were incubated for 72 h at 28 °C. The lowest concentrations without visible growth (as assessed using a binocular microscope) were defined as the MICs. The minimum fungicidal concentrations (MFCs) were determined by serial sub-cultivation of 2 μL in microtitre plates containing 100 μL of malt broth per well and further incubation for 72 h at 28 °C. The lowest concentration with no visible growth was defined as the MFC, indicating 99.5% killing of the original inoculum. 5% DMSO was used as a negative control, while bifonazole and ketoconazole were used as positive controls. Samples were tested in duplicate and experiments were repeated three times.

Toxicity for liver cells. A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house. It was designated as PLP2. Briefly, the liver tissues were rinsed in Hank's balanced salt solution containing 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, and divided into 1×1 mm^3 explants. Some of these explants were placed in 25 cm^3 tissue flasks in DMEM supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, and incubated at 37 °C with a humidified atmosphere containing 5% CO_2. The medium was changed every 2 days. Cultivation of the cells was continued with direct monitoring every 2–3 days using a phase contrast microscope. Before confluence, cells were sub-cultured and plated in 96-well plates at a density of 1.0×10^4 cells per well, and cultivated in DMEM medium with 10% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin.[32] Cells were treated for 48 h with the different diluted sample solutions and the same procedure described in the previous section for the SRB assay was followed. The results were expressed as GI_{50} values (sample concentrations that inhibited 50% of the net cell growth). Ellipticine was used as a positive control.

Statistical analysis

Three samples were used and all the assays were carried out in triplicate. The results are expressed as mean values with standard deviations (SD). Results were compared by means of a Student's *t*-test to determine the significant difference among samples, with $p \leq 0.05$. The analysis was carried out using the SPSS v. 22.0 program (IBM Corp., Armonk, NY, USA).

Results and discussion

Chemical characterization of the *P. ostreatoroseus* formulations

The *P. ostreatoroseus* fruiting body was nutritionally characterized and the results are presented in Table 1. The sample showed regular contents of moisture and ash, besides an exceptional content of total fibre. The basidioma also presented a high content of protein and low fat levels. Patil *et al.*[33] reported a similar value of protein content (21 g/100 g) for *Pleurotus ostreatus*. Fernandes *et al.*[12] found very distinct nutritional values referring to protein and carbohydrate contents for *P. ostreatus*, but similar results referring to moisture and ash contents.

The herein characterized fruiting bodies and the mycelia produced by submerged culture were subjected to an ethanolic extraction in order to prepare bioactive formulations, which were characterized in terms of hydrophilic and lipophilic compounds (Table 2). Regarding the free sugar composition, it was

Table 1 Nutritional characterization of *P. ostreatoroseus* fruiting bodies expressed on a dry weight basis (mean ± SD)

	P. ostreatoroseus fruiting bodies
Fat (g/100 g)	3.0 ± 0.1
Proteins (g/100 g)	26.0 ± 0.2
Ash (g/100 g)	7.6 ± 0.1
Carbohydrates (g/100 g)	18.4 ± 0.1
Total fibre (g/100 g)	45.0 ± 0.2

Table 2 Chemical characterization of *P. ostreatoroseus* fruiting body- and mycelium-based formulations (mean ± SD)

Free sugars	Fruiting body	Mycelium	*t*-Students test *p*-value
Fructose	0.46 ± 0.05	10 ± 1	<0.001
Mannitol	12.0 ± 0.1	15 ± 1	0.006
Sucrose	0.84 ± 0.09	10 ± 1	<0.001
Trehalose	10.3 ± 0.5	15.6 ± 0.3	<0.001
Melezitose	2.44 ± 0.03	7.5 ± 0.4	<0.001
Total (mg g^{-1} extract)	26.0 ± 0.3	58 ± 3	<0.001
Organic acids			
Oxalic acid	12 ± 1	39 ± 6	<0.001
Malic acid	95 ± 1	52 ± 1	<0.001
Citric acid	101 ± 26	0.063 ± 0.002	<0.001
Fumaric acid	4.14 ± 0.01	nd[a]	—
Total (mg g^{-1} extract)	212 ± 25	91 ± 8	<0.001
Phenolic compounds			
p-Hydroxybenzoic acid	0.129 ± 0.001	nd	—
cis p-Coumaric acid	0.03 ± 0.01	nd	—
trans p-Coumaric acid	0.032 ± 0.003	nd	—
Cinnamic acid	0.050 ± 0.002	0.0065 ± 0.0005	<0.001
Total (μg g^{-1} extract)	0.24 ± 0.01	0.0065 ± 0.0005	<0.001
Tocopherols			
α-Tocopherol	0.08 ± 0.00	0.09 ± 0.01	0.083
β-Tocopherol	0.45 ± 0.02	0.41 ± 0.01	0.026
Total (μg g^{-1} extract)	0.53 ± 0.02	0.50 ± 0.01	0.044

[a] nd – not detected.

possible to quantify five distinct compounds, namely fructose, mannitol, sucrose, trehalose, and melezitose (Fig. 2). The mycelium-based formulation revealed higher contents of all identified sugars than the basidioma-based preparation, with a total content of free sugars more than 2-fold higher. This notable difference between the total sugar content presented by the studied formulations could be explained, among other reasons, by the utilization of a sugar-rich wheat bran extract medium to produce the mycelium biomass. Once it is impossible to eliminate all the sugar provided by the culture medium through the filtration and washing processes previously described, the mycelium sample might have assimilated some free sugars from the medium. Reis *et al.*[27] reported lower contents of fructose (0.1 ± 0.00 mg g^{-1} extract and 0.3 ± 0.00 mg g^{-1} extract) and mannitol (5.4 ± 0.04 mg g^{-1} extract and 6.0 ± 0.00 mg g^{-1} extract) for ethanolic extracts of *Pleurotus ostreatus* and *Pleurotus eryngii*, respectively. These authors also found a lower content of sucrose (0.3 ± 0.00 mg g^{-1} extract) in the ethanolic extract of *P. eryngii*. Beluhan and Ranogajec[34] reported a much higher content of mannitol (98.20 ± 0.55 mg g^{-1} extract) and a similar content of trehalose (17.9 ± 0.12 mg g^{-1} extract) for an ethanolic extract prepared from basidiomas of *P. ostreatus*.

It was possible to quantify four different organic acids (Table 2), namely oxalic, malic, citric and fumaric acids. The mycelium-based formulation revealed the highest concentration of oxalic acid, while the fruiting body-based formulation presented the highest contents of malic and citric acids. Fumaric acid was only found in the fruiting body extract, which also presented the highest content of total organic acids. The profile of organic acids described by Fernandes *et al.*[12] for *P. ostreatus* was slightly different, since the authors detected quinic instead of malic acid.

The prepared formulations revealed the presence of *p*-hydroxybenzoic, *cis p*-coumaric and *trans p*-coumaric acids, as well as cinnamic acid (Table 2). *p*-Hydroxybenzoic, *cis p*-coumaric and *trans p*-coumaric acids were only found in the fruiting body-based formulation. Cinnamic acid was found in both formulations, although the fruiting body extract had much the higher content of this compound. The fruiting body-based formulation was clearly richer in phenolic acids compared with the mycelium-based one. Reis *et al.*[28] also reported the presence of *p*-hydroxybenzoic in the basidioma and mycelium of

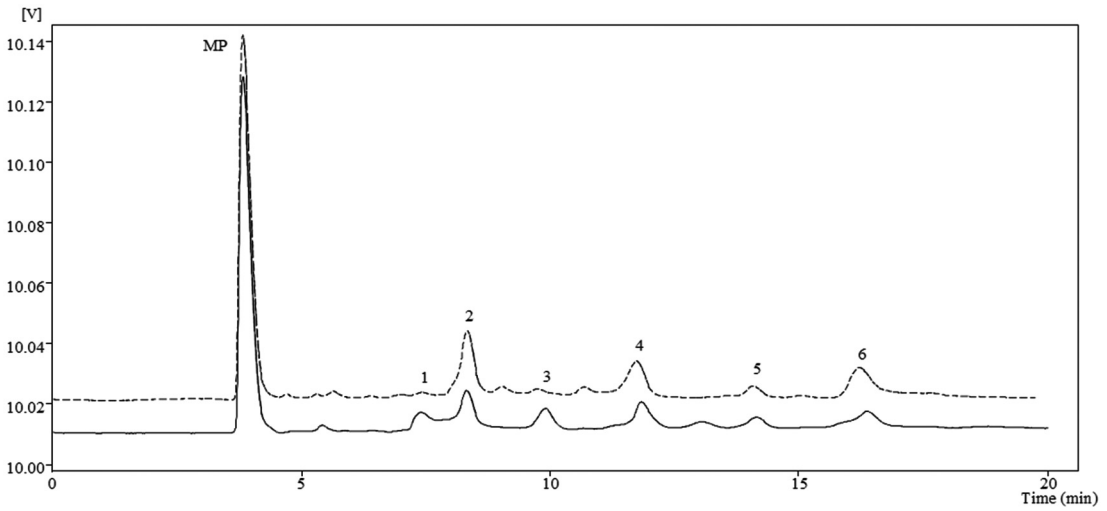

Fig. 2 Individual profiles of *P. ostreatoroseus* fruiting body (––) and mycelium (—) with regard to sugars: 1 – fructose, 2 – mannitol, 3 – sucrose, 4 – trehalose, 5 – melezitose, 6 – raffinose (IS) and MP – mobile phase.

P. ostreatus (1.56 ± 0.06 and 0.05 ± 0.00 l μg g^{-1} dw, respectively), as well as cinnamic acid (0.23 ± 0.02 and 9.65 ± 0.86 μg g^{-1} dw, respectively).

α-Tocopherol and β-tocopherol were found in both formulations, with no statistical differences in the contents of each of these compounds. These two vitamers of tocopherols were also present in the profile of *Pleurotus* species reported by Reis *et al.*[27] and Lin *et al.*[21]

Bioactivity of the *P. ostreatoroseus* formulations

The fruiting body-based formulation revealed higher reducing power, DPPH scavenging activity, β-carotene bleaching inhibition and lipid peroxidation inhibition in brain homogenates than the mycelium-based preparation (Table 3). Reis *et al.*[28] reported lower reducing power (EC_{50} values = 3.31 ± 0.03 mg mL^{-1} and 3.72 ± 0.09 mg ml^{-1}, respectively), DPPH scavenging activity (6.54 ± 0.16 mg mL^{-1} and 8.67 ± 0.12 mg mL^{-1}, respectively), β-carotene bleaching inhibition (EC_{50} values = 2.74 ± 0.16 mg mL^{-1} and 4.68 ± 0.60 mg mL^{-1}, respectively) and TBARS formation inhibition (EC_{50} values 2.58 ± 0.86 mg mL^{-1} and 3.95 ± 0.58 mg mL^{-1}, respectively) for methanolic extracts prepared from basidiomas of *P. ostreatus* and *P. eryngii*. Regarding the DPPH scavenging activity, Tsai *et al.*[35] reported lower activity in ethanolic extracts of *P. ostreatus* from Taiwan (5.58 ± 0.24 mg mL^{-1}). Also, the *P. ostreatoroseus* mycelium ethanolic extract studied herein had higher DPPH scavenging activity than the *P. ostreatus* and *P. eryngii* mycelia methanolic extracts (EC_{50} values 58.13 ± 3.02 mg mL^{-1} and 25.40 ± 0.33 mg mL^{-1}, respectively[28]). The studied ethanolic preparation revealed lower lipid peroxidation inhibition measured by the TBARS assay than the mycelium methanolic extract of *P. ostreatus* (EC_{50} value = 1.08 ± 0.86 mg mL^{-1}), but a much higher inhibition than the mycelium extract of. *P. eryngii* (EC_{50} value = 21.03 ± 0.45 mg mL^{-1}), as reported by Reis *et al.*[28]

Table 3 Bioactivity of *P. ostreatoroseus* fruiting body- and mycelium-based formulations (mean ± SD)[a]

	Fruiting body	Mycelium	*t*-Students test *p*-value
Antioxidant activity (EC_{50} values, mg mL^{-1})			
Reducing power	1.79 ± 0.01	nd	—
DPPH scavenging activity	4.78 ± 0.02	15.62 ± 0.13	<0.001
β-Carotene beaching inhibition	0.40 ± 0.01	7.62 ± 0.25	<0.001
TBARS inhibition	0.29 ± 0.00	2.34 ± 0.08	<0.001
Anti-inflammatory activity (EC_{50} values, μg mL^{-1})			
NO production	229.75 ± 4.25	261.23 ± 8.44	0.011
Hepatotoxicity (GI_{50} values, μg mL^{-1})			
PLP2 growth inhibition	>400	>400	—

[a] Results of antioxidant activity are expressed as EC_{50} values: sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox EC_{50} values: 41 μg mL^{-1} (reducing power), 42 μg mL^{-1} (DPPH scavenging activity), 18 μg mL^{-1} (β-carotene bleaching inhibition) and 23 μg mL^{-1} (TBARS inhibition). Results of anti-inflammatory activity are expressed as EC_{50} values: sample concentration providing 50% inhibition in the production of NO. Dexamethasone EC_{50} value is 16 μg mL^{-1}. Results of hepatotoxicity are expressed as GI_{50} values: sample concentration providing 50% inhibition of the net cell growth. Ellipticine GI_{50} value is 2.3 μg mL^{-1}.

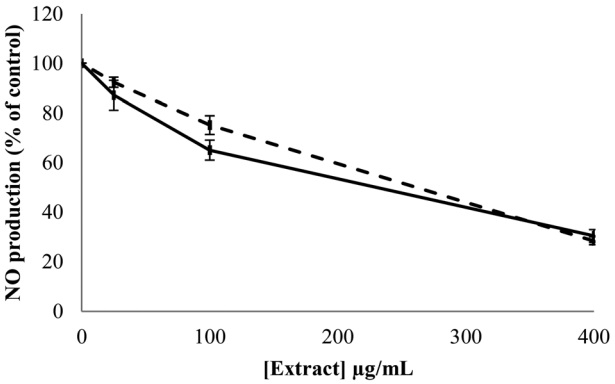

Fig. 3 Nitric oxide production as a function of concentration of *P. ostreatoroseus* fruiting body- (—) and mycelium- (––) based formulations. As the production of nitric oxide is proportional to the inflammatory process, a decrease in the nitric oxide concentration corresponds to potential anti-inflammatory activity.

P. ostreatoroseus formulations revealed a dose-dependent potential anti-inflammatory activity (Fig. 3), with a relevant decrease of NO production even in the presence of lower concentration extracts (up to 400 μg mL^{-1}). The fruiting body-based formulation revealed higher activity (lower EC_{50} value) than the mycelium-based preparation (Table 3). Moro *et al.*[36] investigated the anti-inflammatory activity of a methanolic extract of *P. ostreatus* in LPS-activated macrophages and reported no anti-inflammatory activity. Nonetheless, Lin *et al.*[21] reported anti-inflammatory effects of ethanolic extracts from *P. eryngii* fruiting bodies and correlated these effects with their contents of antioxidant components. Thus, the higher anti-inflammatory activity revealed by the fruiting body-based formulation, when compared to the correspondent mycelium-based preparation, may be justified by its higher contents of hydrophilic and lipophilic antioxidant compounds, including phenolic acids, as also due to its higher antioxidant capacity. In fact, oxidative stress caused by the production of nitric oxide (NO) during inflammation processes has been related to the occurrence of several diseases such as cancer, diabetes, renal disease and arthritis.[37,38] The elimination of NO by NO scavengers or the inhibition of its production by iNOS inactivator alleviates these illness conditions. Thus, the scavenging of NO or suppression of NO production by iNOS are clearly promising indices in the screening of new functional foods.[39,40]

The studied bioactive formulations exhibited antibacterial activity against all bacteria tested (Table 4). The *P. ostreatoroseus* basidioma ethanolic extract presented higher antibacterial activity than the correspondent mycelium extract against *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhimurium* and *Enterobacter cloacae*. The basidioma-based formulation also presented an antibacterial activity similar to the commercial antibiotic

Table 4 Antibacterial and antifungal activities of *P. ostreatoroseus* fruiting body- and mycelium-based formulations[a]

	Fruiting body	Mycelium	Streptomycin	Ampicillin
Bacteria	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC
Staphylococcus aureus	0.30/0.60	0.40/0.75	0.25/0.50	0.10/0.15
Bacillus cereus	0.15/0.30	0.40/0.75	0.05/0.10	0.10/0.15
Listeria monocytogenes	0.60/1.20	0.60/1.20	0.15/0.30	0.15/0.30
Micrococcus flavus	0.60/1.20	0.45/0.60	0.13/0.25	0.10/0.15
Pseudomonas aeruginosa	0.30/1.20	0.60/1.20	0.05/0.10	0.10/0.20
Escherichia coli	0.60/1.20	0.45/0.60	0.05/0.10	0.30/0.50
Salmonella typhimurium	0.15/0.30	0.45/0.60	0.05/0.10	0.15/0.20
Enterobacter cloacae	0.30/0.60	0.60/1.20	0.05/0.10	0.15/0.20
	Fruiting body	Mycelium	Bifonazole	Ketoconazole
Fungi	MIC/MFC	MIC/MFC	MIC/MFC	MIC/MFC
Aspergillus fumigatus	0.60/2.40	0.60/3.60	0.15/0.20	0.20/0.50
Aspergillus versicolor	0.30/2.40	0.60/3.60	0.10/0.20	0.20/0.50
Aspergillus ochracues	0.15/0.30	0.60/1.20	0.15/0.20	1.50/2.00
Aspergillus niger	0.30/1.20	1.20/2.40	0.15/0.20	0.20/0.50
Trichoderma viride	0.15/0.30	0.30/0.60	0.15/0.20	1.0/1.0
Penicillium funiculosum	0.30/0.60	0.60/1.20	0.20/0.25	0.20/0.50
Penicillium ochrochloron	0.60/3.60	0.30/0.60	0.20/0.25	2.50/3.50
P. verrucosum	0.60/3.60	1.20/2.40	0.10/0.20	0.20/0.30

[a] MIC – minimum inhibitory concentration; MBC – minimum bactericidal concentration; MFC – minimum fungicidal concentration.

streptomycin (MIC 0.25 mg mL^{-1}) against *Staphylococcus aureus*. Both studied formulations possessed the same activity against *Listeria monocytogenes*, and it was only in the case of *Micrococcus flavus* that the mycelium-based preparation presented higher antibacterial activity than the fruiting body-based formulation. Alves *et al.*[10] reported high antibacterial activity of an ether extract of *Pleurotus pulmonarius* against *Staphylococcus aureus*. Tambekar *et al.*[41] reported the antimicrobial ability of ethanolic, methanolic and xylene extracts of *P. pulmonarius* against *Escherichia coli* and *Pseudomonas aeruginosa*. Sulphated polysaccharides from *P. eryngii* showed inhibition against *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes*.[42]

Regarding antifungal activity (Table 4), the *P. ostreatoroseus* basidioma ethanolic extract presented higher activity than the correspondent mycelium-based preparation against *Aspergillus versicolor*, *A. ochracues*, *A. niger*, *Trichoderma viride*, *Penicillium funiculosum* and *P. verrucosum*. The studied basidioma ethanolic extract also presented similar antifungal activity to that of the commercial antibiotic bifonazole (0.15 mg mL^{-1}) against *Aspergillus ochracues* and *Trichoderma viride*. Both bioactive formulations possessed the same activity against *Aspergillus fumigatus*. It was only in the case of *Penicillium ochrochloron* that the mycelium-based formulation presented higher antifungal activity than the fruiting body-based preparation. Hearst *et al.*[43] found no antifungal activity in the aqueous extract of *Pleurotus ostreatus* against *Aspergillus fumigatus*, *A. niger* and *Penicillium* sp. Moreover, Wang *et al.*[44] reported the activity of an antifungal peptide isolated from *Pleurotus eryngii* fruiting bodies and Ngai *et al.*[45] reported antifungal effects of a ribonuclease isolated from basidiomas of *P. pulmonarius*. In both cases, the inhibition of mycelium growth was against *Fusarium oxysporum* and *Mycosphaerella arachidicola*.

As the *P. ostreatoroseus* bioactive formulations displayed antioxidant, anti-inflammatory and antimicrobial activity, it was important to guarantee an absence of cytotoxicity against liver cells, which are considered the best *in vitro* model for studies of human cytotoxicity. The studied samples revealed no toxicity in liver primary culture PLP2, since the GI_{50} values obtained were higher than the highest concentration tested (>400 μg mL^{-1}). The positive control ellipticine gave a GI_{50} = 2.29 μg mL^{-1} (Table 3).

Overall, and to the best of our knowledge, this is the first report of anti-inflammatory properties of *P. ostreatoroseus* fruiting body and mycelium extracts, and from the results obtained, a clear anti-inflammatory and antimicrobial potential of the tested samples can be inferred. Therefore, these formulations can be used to prepare dietary supplements for nutraceutical purposes.

Conflict of interest

The authors declare no competing financial interest.

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