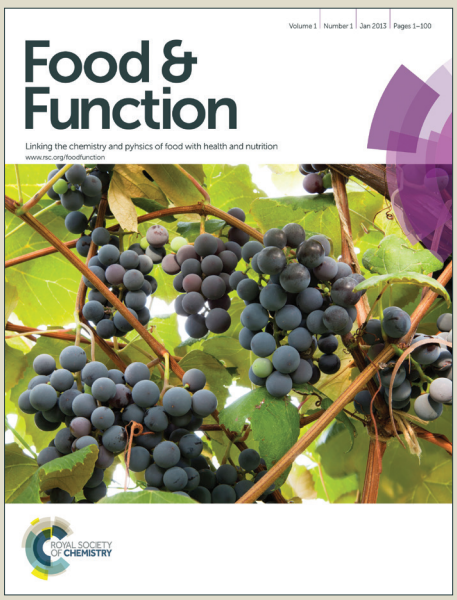
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

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

Rúbia Carvalho Gomes Corrêa[a,b,c], Aloisio Henrique Pereira de Souza[b,c], Ricardo C. Calhelha[a], Lillian Barros[a], Jasmina Glamoclija[d], Marina Sokovic[d], Rosane Marina Peralta[c], Adelar Bracht[c], Isabel C.F.R. Ferreira[a,*]

[a]*Mountain Research Centre (CIMO), ESA, Polytechnic Institute of Bragança, Campus de Santa Apolónia, 1172, 5301-855 Bragança, Portugal*.

[b]*CAPES Foundation, Ministry of Education of Brazil, 70.040-020, Brasília, DF, Brazil.*

[c]*State University of Maringá, Department of Biochemistry, 87020-900, Maringá, PR, Brazil.*

[d]*University of Belgrade, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Bulevar Despota Stefana 142, 11000, Belgrade, Serbia.*

* Author to whom correspondence should be addressed (Isabel C.F.R. Ferreira; e-mail: iferreira@ipb.pt; telephone +351-273-303219; fax +351-273-325405).

ABSTRACT

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 mycelium, obtained from submerged cultivation, 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, besides higher anti-inflammatory and antimicrobial activities, than the mycelium-based preparation. 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.

Keywords: anti-inflammatory activity, antimicrobial activity, bioactive formulations, submerged cultures.

Introduction

Besides their worldwide-appreciated flavour, mushrooms own 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 in their nutraceutical potential[6,7] and bioactivity such as 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 also for 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 delightful flavour of the 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, which 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* (Rumph. ex Fr.), *Pleurotus citrinopileatus* Singer and *Pleurotus eryngii* (DC.) Quél.[9,10,21-23] Nevertheless, to the author's 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 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, along with confirmation of non-toxicity tested 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 latter 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±15ºC. Total fibre was determined by enzymatic-gravimetric method.[24] Carbohydrates value was calculated by difference.

Production of the P. ostretatoroseus mycelia

A commercial isolate of *P. ostreatoroseus* was obtained from a local producer. The stock culture was maintained on wheat bran extract agar slants (**Figure 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 of diameter) into 50 mL medium in a 250 mL Erlenmeyer flask that 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 were boiled in 1 L of distilled water, then the mixture was filtered in gauze and mineral solution[25] at final concentration of 2% was added to the filtrate. For the submerged culture, 150 mL of the same medium were prepared in a 500 mL flask, and pre-culture broth was inoculated (at 1.0 mL/L). 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 freezer and posteriorly freeze-dried.

Preparation of the bioactive formulations

The extraction procedure followed the methodology proposed by Carvajal et al[26], with the choice of ethanol as 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 previously freeze-dried mycelium was 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 n° 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-dried. The freeze-dried powders were stored in 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) 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) (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 and 100 mg/ mL, respectively) were purchased from Gibco Invitrogen Life Technologies (California, USA). Sulforhodamine B, trypan blue, trichloro acetic acid (TCA) and Tris were purchased from Sigma Chemical Co. (Saint Louis, USA). RAW264.7 cells were purchased from ECACC ("European Colletion of Animal Cell Culture") (Salisburg, 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. ostreatosroseus* formulations

Free sugars. The extracts (500 mg) were spiked with the Internal Standard, IS (raffinose, 5 mg/mL), re-dissolved in water (5 mL) and defatted three times with 10 mL of ethyl ether, successively. 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 a high performance liquid chromatograph (HPLC) and the 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 ºC (7971R Grace oven). The mobile phase used was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 mL/min. Sugars 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 ºC. The elution was performed with 3.6 mM sulphuric acid using a flow rate of 0.8 mL/min. The organic acids were quantified by the 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 acids 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 ºC. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic 15% 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% for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm as 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.

194 *Tocopherols.* BHT solution (10 mg/mL, 100 µL) and IS solution (tocol 2 µg/mL, 250
195 µL) were added to the extracts (described above) prior to the extraction procedure. The
196 extracts were homogenized with methanol (4 mL) by vortex mixing (1 min).
197 Subsequently, hexane (4 mL) was added and again vortex mixed for 1 min. After that, a
198 saturated NaCl aqueous solution (2 mL) was added, the mixture was homogenized (1
199 min), centrifuged (5 min, 4000g) and the clear upper layer was carefully transferred to a
200 vial. The sample was re-extracted twice with hexane. The combined extracts were taken
201 to dryness under a nitrogen stream, re-dissolved in 1 mL of hexane, dehydrated with
202 anhydrous sodium sulfate, filtered through a 0.22 µm disposable LC filter disk, and
203 transferred into a dark injection vial. Analysis was performed by HPLC (equipment
204 described above), and a fluorescence detector (FP-2020; Jasco) programmed for
205 excitation at 290 nm and emission at 330 nm, as previously described by the authors.[27]
206 The chromatographic separation was achieved with a Polyamide II (250 × 4.6 mm)
207 normal-phase column from YMC Waters operating at 35 ºC. The mobile phase used
208 was a mixture of hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 ml/min. The
209 compounds were identified by chromatographic comparisons with authentic standards.
210 Quantification was based on the fluorescence signal response of each standard, using
211 the IS (tocol) method and by using calibration curves obtained from commercial
212 standards of each compound. The results were expressed in µg per g of extract.
213
214 **Bioactivity of the *P. ostreatosroseus* formulations**
215 *Antioxidant activity*
216 Successive dilutions of the stock solution were made and used for *in vitro* assays
217 already described by Reis et al[28], to evaluate their antioxidant activity of the samples.
218 The sample concentrations (mg/mL) providing 50% of antioxidant activity or 0.5 of

219 absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages
220 (DPPH, β-carotene/linoleate and TBARS assays) or absorbance at 690 nm
221 (ferricyanide/Prussian blue assay) against the sample concentrations. Trolox was used
222 as a positive control.
223 *Ferricyanide/Prussian blue assay.* The extract solutions with different concentrations
224 (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and
225 potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50°C for 20
226 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was
227 poured into the 48 wells plate in addition to deionized water (0.8 mL) and ferric
228 chloride (0.1% *w/v*, 0.16 mL), and the absorbance was measured at 690 nm in an
229 ELX800 Microplate Reader (Bio-Tek Instruments, Inc; Winooski, VT, USA).
230 *DPPH radical-scavenging activity assay.* This methodology was performed using the
231 Microplate Reader mentioned above. The reaction mixture on the 96 well plate
232 consisted of extract solutions with different concentrations (30 μL) and a methanolic
233 solution (270 μL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to
234 stand for 30 min in the dark, and the absorption was measured at 515 nm. The radical
235 scavenging activity (RSA) was calculated as percentage of the DPPH discoloration
236 using the equation: $\%RSA=[(A_{DPPH}-A_S)/A_{DPPH}]\times100$, where A_S is the absorbance of the
237 solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution.
238
239 *Inhibition of β-carotene bleaching or β-carotene/linoleate assay.* A solution of β-
240 carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two
241 milliliters of this solution were pipetted into a round-bottom flask. The chloroform was
242 removed at 40 °C under vacuum and linoleic acid (40 mg), Tween 80 emulsifier (400
243 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 with 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 2h of assay/ initial absorbance)×100.

Thiobarbituric acid reactive substances (TBARS) assay. Porcine (*Sus scrofa*) brains were obtained from official 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* for10 min. An aliquot (100 μL) of the supernatant was incubated with the different concentrations of the sample solutions (200 μL) in the presence of $FeSO_4$ (10 mM; 100 μL) and ascorbic acid (0.1mM; 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* for10 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 absorbances of the control and the sample solution, respectively.

Anti-inflammatory activity

The extracts were dissolved in water, initially concentrated at 8 mg/mL and then further dilutions were prepared from 8 mg/mL to 0.125 mg/mL. The mouse macrophage-like cell line RAW264.7 was cultured in DMEM medium supplemented with 10% heat-

inactivated foetal 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 x 10^5 cells/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/well and allowed do 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) 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. Hundred microliters of the cell culture supernatant was transferred to the plate in duplicate and mixed with sulfanilamide and NED solutions, 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 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] The fresh overnight culture of bacteria was adjusted spectrophotometrically to a concentration of 1×10^5 CFU/mL. The requested CFU/mL 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 the 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) 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 reduction of a INT color and compared with positive control for each bacterial strains.[30] MBC was determined by serial sub-cultivation of 10 µL into microplates containing 100 µL of TSB. The lowest concentration that shows 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 negative control. Samples were tested in duplicates 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 to a final volume of 100 µL/well. The inocula were stored at 4 °C for further use. Dilutions of the inoculum were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculum.

Minimum inhibitory concentrations (MICs) 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 fungal inoculum. The microplates were incubated for 72 h at 28 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as MIC. The minimum fungicidal concentrations (MFCs) were determined by serial subcultivation 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 ketokonazole were used as positive controls. Samples were tested in duplicates 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 penicillin and 100 µg/mL 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 and 100 U/mL penicillin, 100 mg/mL streptomycin and incubated at 37 ºC 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/well, and cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin.[32] Cells were treated for 48 h with the different diluted sample solutions and the same procedure described in the previous section for SRB assay was followed. The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

Statistical analysis

Three samples were used and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (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 in total fibre. The basidioma also presented 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 submitted to an ethanolic extraction in order to prepare bioactive formulations, which were characterized in terms of hydrophilic and lipophilic compounds (**Table 2**). Regarding free sugars composition, it was possible to quantify five distinct compounds, namely fructose, mannitol, sucrose, trehalose, and melezitose (**Figure 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 contents 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 extract and 0.3 ± 0.00 mg/g extract) and mannitol (5.4 ± 0.04 mg/g extract and 6.0 ± 0.00 mg/g 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 extract) in the ethanolic extract of *P. eryngii*. Beluhan and Ranagajec[34] reported a much higher content of mannitol (98.20 ± 0.55 mg/g extract) and a similar content of trehalose (17.9 ± 0.12 mg/g 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 in 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 in 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 also 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, presenting the fruiting body extract the highest content of this compound. The fruiting body-based formulation was clearly richer in phenolic acids comparing with the mycelium-based one. Reis et al[28] also reported the presence of *p*-hydroxybenzoic in basidioma and mycelium of *P. ostreatus* (1.56 ± 0.06 and 0.05 ± 0.00 l µg/g dw, respectively), besides cinnamic acid (0.23 ± 0.02 and 9.65 ± 0.86 µg/g dw, respectively).

α-Tocopherol and β-tocopherol were found in both formulations, with no statistical differences between them regarding the contents of each one of the compounds. These two vitamers of tocopherols were 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 and 3.72 ± 0.09 mg/ml, respectively), DPPH scavenging activity (6.54 ± 0.16 mg/mL and 8.67 ± 0.12 mg/mL, respectively), β-carotene bleaching inhibition (EC_{50} values = 2.74 ± 0.16 mg/mL and 4.68 ± 0.60 mg/mL, respectively) and TBARS formation inhibition (EC_{50} values 2.58 ± 0.86 mg/mL and 3.95 ± 0.58 mg/mL, respectively) for methanolic extracts prepared from basidiomas of *P. ostratus* e *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). Also, the herein studied *P. ostreatoroseus* mycelium ethanolic extract has higher DPPH scavenging activity than the *P. ostreaus* and *P. eryngii* mycelia methanolic extracts (EC_{50} values 58.13 ± 3.02 mg/mL and 25.40 ± 0.33 mg/mL, respectively[28]). The studied ethanolic preparation revealed lower lipid peroxidation inhibition measured by the T-BARS assay than the mycelium methanolic extract of *P. ostreatus* (EC_{50} value = 1.08 ± 0.86 mg/mL), but a much higher inhibition than the mycelium extract of. *P. eryngii* (EC_{50} value = 21.03 ± 0.45 mg/mL), as reported by Reis et al[28].

P. ostreatoroseus formulations revealed a dose-dependent potential anti-inflammatory activity (**Figure 3**), with a relevant decrease of NO production even in the presence of low concentrated extracts (up to 400 µg/mL). 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 in 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 in 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 screening 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 streptomycin (MIC 0.25 mg/mL) against *Staphylococcus aureus*. Both studied formulations possessed the same activity against *Listeria monocytogenes*, and only in the case of *Micrococcus flavus* 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*. Tambeker 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 the 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 similar to that of the commercial antibiotic bifonazole (0.15 mg/mL) against *Aspergillus ochracues* and *Trichoderma viride*. Both bioactive formulations possessed the same activity against *Aspergillus fumigatus*. Only in the case of *Penicillium ochrochloron* the mycelium-based formulation presented higher antifungal activity than the fruiting body-based preparation. Hearts 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, once the GI_{50} values obtained were higher than the highest concentration tested (> 400 µg/mL). The positive control ellipticine gave a GI_{50} = 2.29 µg/mL (**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

493 results obtained, it can be inferred a clear anti-inflammatory and antimicrobial potential
494 of the tested samples. Therefore, these formulations can be used to prepare dietary
495 supplements with nutraceutical purposes.
496
497 **Competing interests**
498 The authors declare no competing financial interest.
499
500 **Acknowledgements**
501 The authors are grateful to Foundation for Science and Technology (FTC, Portugal) for
502 financial support to CIMO (Pest-OE/AGR/UI0690/2014), L. Barros research contract
503 ("Compromisso para a Ciência") and R.C. Calhelha grant (SFRH/BPD/68344/2010),
504 and to the Serbian Ministry of Education, Science and Technological Development for
505 the Grant No 173032. R.C.G. Correa thanks CAPES Foundation, Ministry of Education
506 of Brazil (CAPES fellow, process number BEX 3974/14-6). R.M. Peralta and A. Bracht
507 are Research Fellows of CNPq (Conselho Nacional de Desenvolvimento Científico e
508 Tecnológico). The authors also thank to A. Fernandes for all the support in some of the
509 laboratorial work.
510
511 **References**
512 1 R.P.Z. Furlani and H.T. Godoy, Rev. *Inst. Adolfo Lutz*, 2005, 64, 149-154.
513 2 I.C.F.R. Ferreira, S.A. Heleno, F.S. Reis, D. Stojkovicd, M.J.R.P. Queiroz, M.H.
514 Vasconcelos and M. Sokovic, *Phytochem*., 2014. In press.
515 doi:10.1016/j.phytochem.2014.10.011.
516 3 S.A. Heleno, L. Barros, M.J. Sousa, A. Martins and I.C.F.R. Ferreira, *Food*
517 *Chem*., 2010, 119, 1443-1450.

4 J.A. Vaz, L. Barros, A. Martins, J.S. Morais, M.H. Vasconcelos and I.C.F.R. Ferreira, *LWT*, 2011, 44, 343-346.

5 L. Barros, C. Pereira and I.C.F.R. Ferreira, *Food Anal. Method*., 2013, 6, 309-316.

6 L. Barros, P. Baptista, D.M. Correia, S. Casal, B. Oliveira and I.C.F.R. Ferreira, *Food Chem.*, 2007, 105, 140-145.

7 L. Barros, T. Cruz, P. Baptista, L.M. Estevinho and I.C.F.R. Ferreira, *Food Chem. Toxicol.*, 2008, 46, 2742-2747.

8 M. Elmastas, O. Isildak, I. Turkekul and N. Temur, *J. Food Compos. Anal.*, 2007, 20, 337-345.

9 I.C.F.R. Ferreira, L. Barros and R.M.V. Abreu, *Curr. Med. Chem.*, 2009, 16, 1543-1560.

10 M.J. Alves, I.C.F.R. Ferreira, J. Dias, V. Teixeira, A. Martins and M. Pintado *Planta Med.*, 2012, 78, 1707-1718.

11 J.C.P. Cardoso, P.L.M.M. Demenjour and M.F. Paz, *Evidência*, 2013, 13, 31-40.

12 A. Fernandes, L. Barros, A. Martins, P. Herbert and I.C.F.R. Ferreira, *Food Chem.*, 2015, 169, 396-400.

13 G.C.S. Bazanella, D.F. De Souza, R. Castoldi, R.F. Oliveira, A. Bracht and R.M. Peralta, *Folia Microbiol.*, 2013, 58, 641-647.

14 C. Ramos, M. Sapata, A. Ferreira, L. Andrada and M. Candeias, *Rev. Cien. Agr.*, 2011, 34, 57-64.

15 C.P. Pokhrel, N. Kalyan, U. Budathoki and R.K.P.Yadav, *Int. J. Agric. Pol. Res.*, 2013, 1, 19–23.

16 R. Singer, Publications of the Mycology Institute of the University of Recife, 1961, 304, 3–26

17 F.R. Rosado, C. Kemmelmeier and S.M.G. Da Costa. J. Basic Microbiol., 2002, 42, 37-44.

18 E.R. Carbonero, A.H.P. Gracher, F.R. Smiderle, F.R. Rosado, G.L. Sassaki, P.A.J. Gorin and M. Iacomini, *Carbohydr Polym*., 2006, 66, 252-257.

19 E.R. Carbonero, A.H.P. Gracher, M.C.C. Rosa, G. Torri, G.L. Sassaki, P.A.J. Gorin and M. Iacomini, *Phytochem*, 2008, 69, 252-257.

20 A.H.P. Gracher, T.R. Cipriani, E.R. Carbonero, P.A.J. Gorin and M. Iacomini, *Thrombosis Res.*, 2010, 126, e180-e187.

21 J.-T. Lin, C.-W. Liu, H.-C. Chen, C.-C. Hu, L.-D. Juang, C.-C. Shiesh, and D.-J. Yang, *LWT*, 2014, 55, 374-382.

22 M.L.L. Silveira, F.R. Smiderle, C.P. Moraes, D.G. Borato, C.H. Baggio, Ruthesa, A.C.E. Wisbeck, G.L. Sassaki, T.R. Cipriani, S.A. Furlan and M. Iacominia, *Carbohydrate Polymers*, 2014, 113, 588-596.

23 M.L.L. Silveira, F.R. Smiderle, F. Agostini, E.M. Pereira, M. Bonatti-Chavesa, E. Wisbeck, A.C. Ruthes, G.L. Sassaki, T.R. Cipriani, S.A. Furlan and M. Iacomini, *Inter. J. Bio. Macrom.*, 2015, 75, 90-96.

24 AOAC, Association of Official Analytical Chemists, 16th ed., Arlington VA, USA, 1995.

25 H.A. Vogel, *Microbial Gen. Bull.*, 1956, 13, 578-584.

26 A.E.S.S. Carvajal, E.A. Koehnlein, A.A. Soares, G.J. Eler, A.T.A. Nakashima, A. Bracht and R.M. Peralta, *LWT*, 2012, 46, 493-499.

27 F.S. Reis, L. Barros, A. Martins and I.C.F.R. Ferreira, *Food Chem. Toxicol.*, 2012, 50, 191-197.

28 F.S. Reis, A. Martins, L. Barros and I.C.F.R. Ferreira, *Food Chem. Toxicol.*, 2012, 50, 1201-1207.

29 A. Espinel-Ingroff, *J. Clin. Microbiol.*, 2001, 39, 1360-1367.

30 CLSI, Approved standard, 8th ed. CLSI publication M07-A8. Clinical and Laboratory Standards Institute, Wayne, PA, 2009.

31 C. Booth, Fungal culture media. In J.R. Norris and D.W. Ribbons (Eds.), Methods in microbiology (pp. 49-94). London and New York: Academic Press, 1971.

32 R.M.V. Abreu, I.C.F.R. Ferreira, R.C. Calhelha, R.T. Lima, M.H. Vasconcelos, F. Adega, R. Chaves and M.J.R.P. Queiroz, *Eur. J. Med. Chem.*, 2011, 46, 5800-5806.

33 S.S. Patil, S.A. Ahmed, S.M. Telang and M.M.V. Baig, *Innov. Rom. Food Biotechnol.* 2010, 7, 66-76.

34 S. Beluhana and A. Ranogajec, *Food Chem.*, 2011, 124, 1076–1082.

35 S.-Y. Tsai, S.-J. Huang, S.-H. Lo, T.-P. Wu, P.-Y. Lian and J.-L. Mau, *Food Chem.*, 2009, 113, 578–584.

36 C. Moro, I. Palacios, M. Lozano, M. D'Arrigo, E. Guillamón, A. Villares, J.A. Martínez and A. García-Lafuente, *Food Chem.*, 2012, 130, 350-355.

37 J.F. Comar, A.B. Sá-Nakanishi, A.L. Oliveira, M.M.N. Wendt, C.A. Bersani-Amado, E.L. Ishii-Iwamoto, R.M. Peralta and A. Bracht, *Free Rad. Biol. Med.*, 2013, 58, 144-153.

38 M.N.N. Wendt, A.B. Sá-Nakanishi, C.V.C. Ghizoni, C.A. Bersani-Amado, R.M. Peralta, A. Bracht and J.F. Comar, *Exp. Mol. Pathol.*, 2015, 98, 549-557.

39 P.J. Tsai, T.-H. Tsai, C.-H. Yu and S.-C. Ho., *Food Chem.*, 2007, 103, 181–187.

40 M.J. Rebelo, C. Sousa, P. Valentão, R. Rego and P.B. Andrade, *Food Chem.*, 2014, 163, 16-22.

41 D.H. Tambekar, T.P. Sonar, M.V. Khodke and B.S. Khante, *Int. J. Pharmacol.*, 2006, 2, 584-587.

42 S. Li and N.P. Shah, *Food Chem.*, 2014, 165, 262-270.

43 R. Hearst, D. Nelson, G. McCollum, B.C. Millar, Y. Maeda, E.C. Goldsmith, P.J. Rooney, A. Loughrey, J.R. Rao and J.E. Moore, *Comp. Ther. Clin. Pract.*, 2009, 15, 5-7.

44 H. Wang and T.B. Ng., Peptides, 2004, 25, 1-5.

45 P.H.K. Ngai and T.B. Ng, Peptides, 2004, 25, 11-17.

Table 1. Nutritionally 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 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	-
Total (mg/g 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 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 extract)	0.53 ± 0.02	0.50 ± 0.01	0.044

nd- not detected.

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

	Fruiting body	Mycelium	*t*-Students test *p*-value
Antioxidant activity (EC_{50} values, mg/mL)			
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)			
NO production	229.75 ± 4.25	261.23 ± 8.44	0.011
Hepatotoxicity (GI_{50} values, µg/mL)			
PLP2 growth inhibition	>400	>400	-

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

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

Bacteria	Fruiting body MIC/MBC	Mycelium MIC/MBC	Streptomycin MIC/MBC	Ampicillin 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
Fungi	Fruiting body MIC/MFC	Mycelium MIC/MFC	Bifonazole MIC/MFC	Ketoconazole 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 ochrochlron	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

MIC- minimum inhibitory concentration; MBC- minimum bactericidal concentration; MFC- minimum fungicidal concentration.

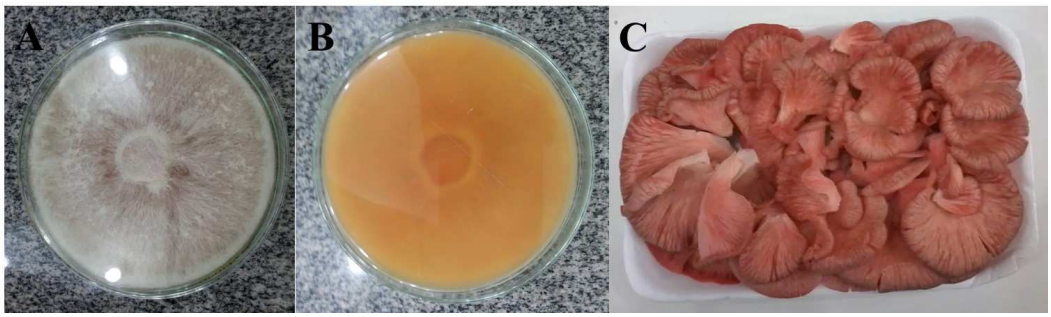


Figure 1. Morphological characteristics of *P. ostreatoroseus*. **a.** Mycelium cultivated on wheat bran extract agar slants. **b.** Bottom of the mycelium plate of figure a in which it can be observed the characteristical rosy coloration. **c.** Fruiting bodies of commercial package.

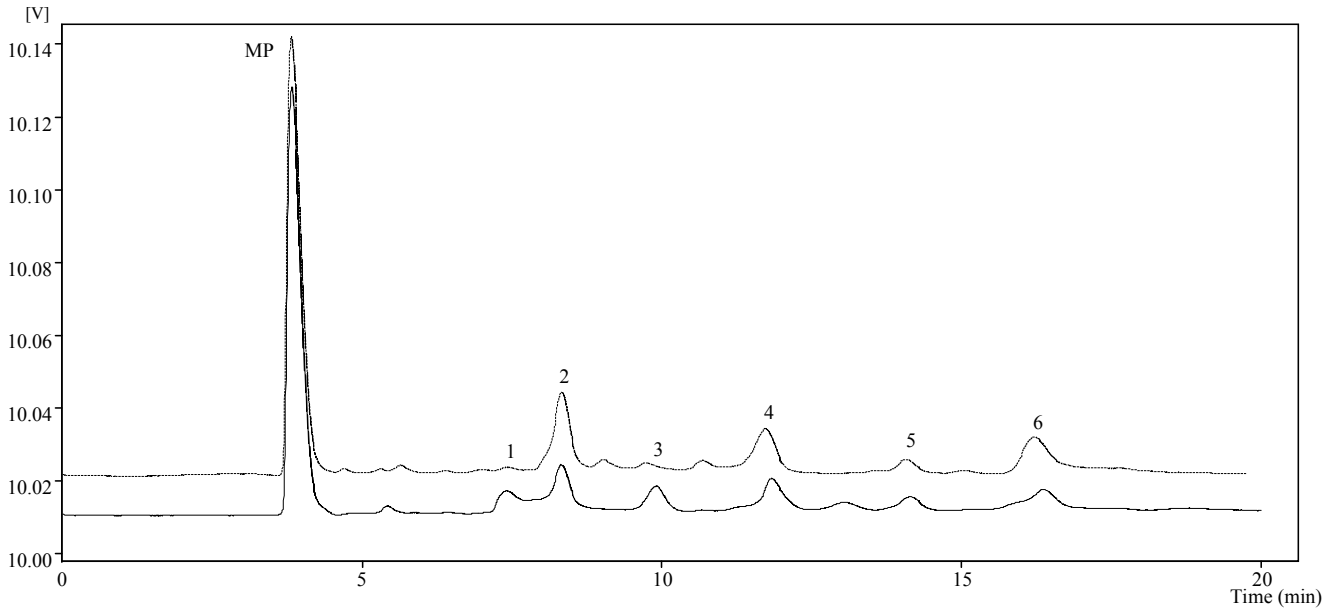


Figure 2. Individual profile of *P. ostreatoroseus* fruiting body (----) and mycelium (—) in sugars: 1- fructose, 2- mannitol, 3- sucrose, 4- trehalose, 5- melezitose, 6- raffinose (IS) and MP- mobile phase.

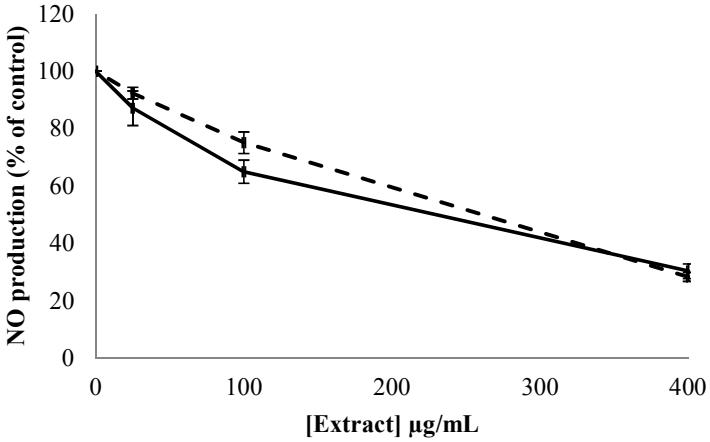
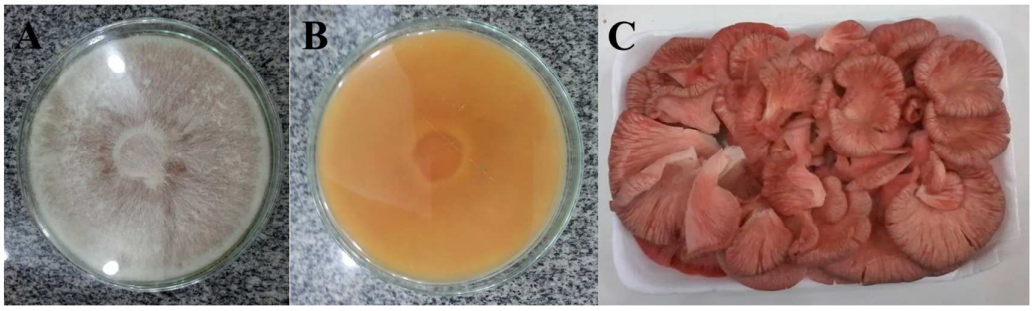


Figure 3. Nitric oxide production as function of concentration of *P. ostreatoroseus* fruiting body (——) and mycelium (----) based formulations. As the production of oxide nitric is proportional to the inflammatory process, a decrease in the nitric oxide concentration corresponds to potential anti-inflammatory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

Graphical Abstract

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

Rúbia Carvalho Gomes Corrêa, Aloisio Henrique Pereira de Souza, Ricardo C. Calhelha, Lillian Barros, Jasmina Glamoclija, Marina Sokovic, Rosane Marina Peralta, Adelar Bracht, Isabel C.F.R. Ferreira



Fruiting body and mycelium of *Pleurotus ostreatoroseus* were used to prepare bioactive formulations.
