A comparative study of chemical composition, antioxidant and antimicrobial properties of *Morchella esculenta* (L.) Pers. from Portugal and Serbia

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ABSTRACT

A comparative study on chemical composition (nutritional value, primary and secondary metabolites), antioxidant properties (scavenging activity, reducing power and inhibition of lipid peroxidation), and antimicrobial activity (antibacterial and demelanizing properties) of two samples of *Morchella esculenta* (morel) from different countries (Portugal and Serbia) was performed. This species was chosen for being one of the most highly prized edible mushrooms in the world. Both samples are rich in carbohydrates (including free sugars) and proteins, and contain several bioactive compounds such as organic acids, phenolic compounds and tocopherols. Polyunsaturated fatty acids were the most abundant compounds followed by mono or saturated fatty acids. Sample from Portugal (SP) gave higher radical scavenging activity and reducing power, while sample from Serbia (SS) showed higher lipid peroxidation inhibition. Both samples gave antibacterial activity against five bacteria (in some cases even better than standard antibiotics) and demelanizing activity against four micromycetes, showing SS higher activities. As far as we know, this is the first study reporting chemical compounds and bioactivity of morel samples from Portugal and Serbia. Furthermore, a novel method for evaluation of demelanizing activity was presented.

Keywords: Morchella esculenta; Nutrients; Antioxidants; Antibacterial activity; Demelanizing activity.

1. Introduction

Mushrooms contain a huge diversity of biomolecules with nutritional (Kalac, 2009) and/or bioactive properties (Ferreira, Barros, & Abreu, 2009; Ferreira, Ferreira, Vaz, Vasconcelos, & Martins 2010; Alves et al., 2012). Due to these properties, they have been recognized as functional foods, and a valuable source of natural medicines and nutraceuticals. Morel species are reported to minimize oxidative damage in organisms that occurs in several chronic diseases (Ferreira et al., 2009). Furthermore, these species can be used to find new antimicrobials overlapping the bacterial resistance to first choice antibiotics (Alves et al., 2012). Phenolic compounds, tocopherols and organic acids are considered to be the most responsible for antioxidant activity of mushrooms (Ferreira et al., 2009; Reis et al., 2012; Leal et al., 2013). On the other hand, low molecular weight compounds found in the mushrooms such as sesquiterpenes and other terpenes, steroids, anthraquinones, benzoic acid derivatives, quinolines, and oxalic acid, but also high molecular weight compounds such as peptides and proteins have been reported to possess antimicrobial activity (Alves et al., 2012).

Morchella esculenta (L.) Pers. (morel) is one of the most widely appreciated wild edible mushrooms. Since commercial cultivation of this mushroom has not been successful till now, its cultured mycelium is extensively used as a flavouring agent. Recently, it has been proven that morel possess anti-inflammatory, antitumor, antioxidant and antimicrobial activities (Mau, Chang, Huang, & Chen, 2004; Nitha, Meera, & Janardhanan, 2007; Nitha and Janardhanan, 2008; Nitha, Fijesh, & Janardhanan, 2011; Alves et al., 2012).

Steroids (mainly ergosterol derivatives) and polysaccharides isolated from *M. esculenta* were reported to possess both *in vitro* and *in vivo* antioxidant and NF-kappa B inhibiting properties (Meng et al., 2010; Kim, Lau, Tay, & Blanco, 2011).

Furthermore, galactomannan was also isolated from *M. esculenta* and showed immunostimulatory properties (Duncan et al., 2002).

There are a few reports on nutritional value of *M. esculenta* fruiting bodies from Pakistan (Wahid, Sattar, & Khan, 1988), on antioxidants of specimens from Turkey (Elmastas et al., 2006) and Spain (Ramírez-Anguiano, Santoryo, Reglero, & Soler-Rivas, 2007), and on antimicrobial activity of *M. esculenta* mycelia from Turkey (Kalyoncu, Oskay, Saglam, Erdogan, & Tamer, 2010). Nevertheless, as far as we know, there are no reports on morel samples from Portugal or Serbia; this is also the first report on fatty acids, organic acids and phenolic compounds composition in *M. esculenta*. Therefore, the present study aimed to provide more detailed investigation on chemical composition and bioactive properties (antioxidant and antimicrobial) of this species. Furthermore, a novel method for evaluation of demelanizing activity is presented.

2. Materials and methods

2.1. Samples

Specimens of *Morchella esculenta* (L.) Pers. were collected in Bragança (Northeast Portugal) and Jabučki rid (Northern Serbia) during November of 2011 and April 2012, respectively. The authentications were done by Dr. Anabela Martins (Polytechnic Institute of Bragança) and Dr. Jasmina Glamočlija (Institute for Biological Research, Belgrade). Voucher specimens were deposited at herbarium of School of Agriculture of Polytechnic Institute of Bragança, Portugal, and at Fungal Collection Unit of the Mycological Laboratory, Department for Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia, respectively.

The specimens were immediately dried by lyophilisation (FreeZone 4.5, Labconco, Kansas, USA and LH Leybold, Lyovac GT2, Frenkendorf, Switzerland, respectively), reduced to a fine dried powder (20 mesh), mixed to obtain an homogenate sample and kept at -20 ºC until further analysis.

2.2. Standards and Reagents

Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also other individual fatty acid isomers and standards of sugars (D-(-)-fructose, D-(+)-mannitol, D-(+)-trehalose), tocopherols (α-, β-, δ- and γ-isoforms), organic acids (citric acid, malic acid, oxalic acid, fumaric acid and quinic acids), phenolic compounds (gallic, *p*-hydroxybenzoic, *p*-coumaric and protocatechuic acids), and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Racemic tocol, 50 mg/mL, was purchased from Matreya (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA). Mueller–Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia). Streptomycin and ampicillin were from Sigma and purchased from Galenika and Panfarma (Belgrade, Serbia), respectively.

2.3. Chemical composition

2.3.1. Nutritional value

The samples were analysed for chemical composition (moisture, proteins, fat, carbohydrates and ash) using the AOAC procedures (AOAC, 1995). The crude protein

content (N × 4.38) of the samples 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 ºC. Total carbohydrates were calculated by difference. Energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein + g carbohydrate) + 9 × (g fat).

2.3.2. Sugars

Free sugars were determined by a High Performance Liquid Chromatography (HPLC) system consisted of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000) and auto-sampler (AS-2057 Jasco), coupled to a refraction index detector (RI detector Knauer Smartline 2300) as previously described by the authors (Heleno, Barros, Sousa, Martins, & Ferreira, 2009). Sugars were identified 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 commercial standards of each compound. The results were expressed in g per 100 g of dry weight.

2.3.3. Fatty Acids

Fatty acids were determined after a transesterification procedure as described previously by the authors (Heleno et al., 2009), using a gas chromatographer (DANI 1000) equipped with a split/splitless injector and a flame ionization detector (GC-FID). Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using CSW 1.7

software (DataApex 1.7). The results were expressed in relative percentage of each fatty acid.

2.3.4. Tocopherols

Tocopherols were determined following a procedure previously optimized and described by the authors (Heleno, Barros, Sousa, Martins, & Ferreira, 2010). 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. 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 mg per 100 g of dry weight.

2.3.5. Carotenoids

β-carotene and lycopene were determined following a procedure previously described by Nagata & Yamashita (1992). A fine dried powder (500 mg) was vigorously shaken with 10 mL of acetone–hexane mixture (4:6) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance (A) of the filtrate was measured at 453, 505, 645 and 663 nm. Content of β-carotene and lycopene were calculated according to the following equations:

(1) β-carotene (mg/100 mL) = $0.216\times A_{663} - 1.220\times A_{645} - 0.304\times A_{505} + 0.452\times A_{453}$;

(2) Lycopene (mg/100 mL) = $-0.0458\times A_{663} + 0.204\times A_{645} - 0.304\times A_{505} + 0.452\times A_{453}$;

and further expressed in mg per 100 g of dry weight (dw).

2.3.6. Organic acids

Organic acids were determined following a procedure previously optimized and described by the authors (Reis et al., 2012). Analysis was performed by ultra fast liquid chromatograph (UFLC) coupled to photodiode array detector (PDA), using a Shimadzu 20A series UFLC (Shimadzu Corporation). Detection was carried out in a PDA, using 215 nm and 245 as preferred wavelengths. 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 g per 100 g of dry weight.

2.3.7. Phenolic compounds

Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA) as previously described by the authors (Heleno et al., 2012). Double online detection was carried out in the diode array detector (DAD) using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were characterized according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared from different standard compounds. The results were expressed in g per 100 g of dry weight.

2.4. Extracts preparation for evaluation of bioactive properties

Samples (~5 g) were extracted by stirring with 150 mL of methanol (25ºC at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with an additional portion of methanol. The combined methanolic extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210; Flawil,

Switzerland) to dryness. The extract was redissolved in i) methanol for antioxidant activity assays or ii) 5% DMSO containing 0.02% Tween 80 for antimicrobial activity assays.

2.5. Antioxidant activity

2.5.1. General

Successive dilutions of the stock solution were made and submitted to *in vitro* assays already described by the authors to evaluate the antioxidant activity of the samples. The sample concentrations providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene/linoleate and TBARS assays) or absorbance at 690 nm (reducing power assay) against sample concentrations. The commercial standard trolox was used as positive control.

2.5.2. Folin-Ciocalteu assay

One of the extract solutions (5 mg/mL; 1 mL) was mixed with *Folin-Ciocalteu* reagent (5 mL, previously diluted with water 1:10, v/v) and sodium carbonate (75 g/L, 4 mL). The tubes were vortex mixed for 15 s and allowed to stand for 30 min at 40°C for colour development. Absorbance was then measured at 765 nm (Analytikjena spectrophotometer; Jena, Germany) (Singleton and Rossi, 1965). Gallic acid was used to obtain the standard curve (0.0094 – 0.15 mg/mL), and the reduction of *Folin-Ciocalteu* reagent by the samples was expressed as mg of gallic acid equivalents (GAE) per g of extract.

2.5.3. Ferricyanide/Prussian blue assay

The extract solutions with different concentrations (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50ºC for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48 wells plate, as also deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm in ELX800 Microplate Reader (Bio-Tek Instruments, Inc; Winooski, USA) (Barros, Heleno, Carvalho, & Ferreira, 2010). The reducing power was obtained directly from the absorbances.

2.5.4. DPPH scavenging activity assay

The methodology used was adapted from Brand-Williams, Cuvelier, & Berset (1995) but using the Microplate Reader mentioned above. The reaction mixture on 96 wells plate consisted of a solution by well of the extract solutions with different concentrations (30 µL) and methanolic solution (270 µL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 30 min in the dark, and the absorption was measured at 515 nm (Barros et al., 2010). The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration 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.

2.5.5. β-carotene/linoleate assay

A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40ºC 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 with different concentrations (0.2 mL). The tubes were shaken and incubated at 50ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm (Mi-Yae, Tae-Hun, & Nak-Ju, 2003). β-Carotene bleaching inhibition was calculated using the following equation: (β-carotene content after 2h of assay/initial β-carotene content) × 100.

2.5.6. TBARS assay

Porcine (*Sus scrofa*) brains were obtained from official slaughtering animals, dissected, and homogenized with a Polytron in ice cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 w/v brain tissue homogenate which was centrifuged at 3000*g* for10 min. An aliquot (100 µL) of the supernatant was incubated with the different concentrations of the samples solutions (200 µL) in the presence of $FeSO_4$ (10 mM; 100 µL) and ascorbic acid (0.1 mM; 100 µL) at 37ºC for 1 h. The reaction was stopped by the addition of trichloro acetic acid (28% w/v, 500 µL), followed by thiobarbituric acid (TBA,2%, w/v, 380 µL), and the mixture was then heated at 80ºC for 20 min. After centrifugation at 3000*g* for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm (Ng, Liu, & Wang, 2000). The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A - B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively.

2.6. Antibacterial activity

2.6.1. Bacteria strains

Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (human isolate) and Gram-positive bacteria: *Listeria monocytogenes* (NCTC 7973) and *Staphylococcus aureus* (ATCC 6538) were used. The microorganisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research 'Siniša Stankovic', Belgrade, Serbia.

2.6.2. Disc-diffusion method

To evaluate extracts antibacterial activity, disc diffusion method was carried out using 6 mm filter discs (Verpoorte, Beek, Thomassen, Aandewiel, & Svendsen, 1983). Bacteria were cultured overnight at 37°C in Tryptic Soy Broth (TSB) medium and then adjusted with sterile saline to a concentration of 1.0×10^5 cfu/mL. The suspension was added to the top of the agar plates in Petri dishes (300 µL/agar plate) with Mueller-Hinton agar and resuspended. Filter discs with extracts (10 µL/disc) were placed on agar plates. After 24 h of incubation at 37°C the diameter of the growth inhibition zones was measured. Streptomycin was used as a positive control (Sokovic, Glamoclija, Marin, Brkic, & Griensven, 2010).

2.6.3. Microdilution test

The antibacterial activity was also evaluated by the microdilution method (Hanel and Raether, 1988; Espinel-Ingroff, 2001). The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0×10^5 CFU/mL. The inocula were prepared daily and stored at +4° C until use. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum.

The minimum inhibitory and bactericidal concentrations (MICs and MBCs) were determined using 96-well microtitre plates. Extracts to be investigated were dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) (5 mg/mL) and added in TSB medium (100 µL) with bacterial inoculum (1.0×10^5 CFU/well) to achieve the wanted concentrations. The microplates were incubated at Rotary shaker (160 rpm) for 24 h at 37° C. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The MBCs were determined by serial sub-cultivation of 5 µL into microtitre plates containing 95 µL of broth per well and further incubation for 24 h. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. The optical density of each well was measured at 655 nm by ELISA microplate reader (Bio-Rad Laboratories, Hercules, CA) and the results were processed with Microplate manager 4.0 (Bio-Rad Laboratories, Hercules, CA) and compared with a blank and with positive control. Streptomycin and ampicillin were used as positive controls (1 mg/mL in sterile physiological saline) (Sokovic et al., 2010). Solution of 5% DMSO was used as negative control.

2.7. Novel method for demelanizing activity using micromycetes

To evaluate extracts demelanizing activity, four microfungi were used: *Aspergillus fumigatus* (ATCC 1022), *Aspergillus flavus* (ATCC 9643), *Penicillium funiculosum* (ATCC 36839) and *Penicillium ochrochloron* (ATCC 9112). The micromycetes were maintained on malt agar and the cultures were stored at 4° C; 96-well microliter plates were used. 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 proximate concentration of 1.0×10^5 in a final volume of 100 µL/well. Dilutions of the inocula were cultured on malt agar to verify the absence of

contamination and to check the validity of the inoculum. Determination of minimum demelanizing concentrations (MDC) was performed by a serial dilution technique. The extracts were dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) (40 mg/mL) and added in broth Malt medium with inoculum. The microplates were incubated at Rotary shaker (160 rpm) for 72 h at 28° C. A sample of mycelium was taken from the periphery of a colony grown on Malt extract medium enriched with different concentrations of *M. esculenta* extracts. The samples were dyed and fixed with lactophenol and observed under a light microscope (Mikroskop DMLS Typ 020 518 500. Leica, Wetzlar. Neubauer Zählkammer. Eppendorf, Hamburg, Germany) to examine structural abnormalities. The lowest concentration that provoked demelanization of fungal hyphae and conidia was determined as MDC. Samples from the control plate without added extracts were also stained and observed. Solution of 5% DMSO was used as a negative control.

2.8. Statistical analysis

Three specimens were used and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 18.0 program.

3. Results and discussion

3.1. Chemical composition

The results of the chemical composition of SP and SS are shown in **Tables 1 and 2** and report nutritional value, primary and secondary metabolites. Carbohydrates were the most abundant macronutrients, followed by proteins and ash. Fat contents were low and

similar in both samples. The energetic contribution of SS was slightly higher due to the higher contribution of carbohydrates (**Table 1**). The nutritional value found herein is also different, particularly in protein levels, from Pakistan specimens (Wahid et al., 1988): protein 32.7%, fat 2.0%, fibre 17.6%, ash 9.7% and carbohydrates 38.0%.

Mannitol and trehalose were found in both samples, while fructose was only detected on SP (**Table 1**). This sample gave the highest levels of total sugars and mannitol, the most abundant polyol in mushrooms (Kalac, 2009), while SS revealed the highest levels of trehalose, a storage disaccharide also common in Basidiomycetes (Koide, Shumway, & Stevens, 2000).

Concerning the fatty acids composition (**Table 1**), polyunsaturated fatty acids (PUFA) predominated over saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). The fatty acids determined in higher percentages, were linoleic acid (C18:2n6); oleic (C18:1n9) or palmitic (C16:0) acids, depending on the sample. Curiously, the essential fatty acid α-linolenic acid (C18:3n3) was quantified in higher amounts in SS. Up to 24 fatty acids were identified and quantified.

Regarding tocopherols, α-, γ- and δ-tocopherols were found in both samples, but SS contained higher total tocopherols content than SP, and revealed a higher levels of γ- and δ-tocopherol (**Table 2**). The presence of this isoform was also reported by Mau et al. (2004) in *M. esculenta* mycelium from Taiwan. Tocopherols are important fat-soluble antioxidants, acting in the cellular membrane; due to their role as scavenger of free radicals act to protect human cells against degenerative malfunctions (Kamal-Eldin & Appelqvist, 1996). Only lycopene, another known antioxidant, was observed in the analyzed samples, and was higher in SS (**Table 2**). β-Carotene was not found neither in the studied samples nor in *M. esculenta* mycelium analyzed by Mau et al. (2004).

Among organic acids, it was possible to quantify oxalic and fumaric acids in both samples. The first mentioned organic acid might have toxicity effects (Nagarajkumar, Jayaraj, Muthukrishnan, Bhaskaran, & Velazhahan, 2005), while fumaric acid possesses interesting biological effects such as anti-inflammatory, neuroprotective, chemopreventive and antimicrobial activity (Baati, Horcajada, Gref, Couvreur, & Serre, 2011). Malic acid was also found in SP, while quinic and citric acids were only found in SS (**Table 2**). The latter present higher amounts of total organic acids. The content of these compounds in food not only influences their flavor, but also their stability, nutrition, and acceptability (Bhandari & Kawabata, 2004).

Two phenolic acids, protocatechuic and *p*-hydroxybenzoic acids were found in both samples (**Table 2**). *p*-Coumaric acid was quantified in SP. Phenolic compounds exhibit a wide range of physiological properties such as anti-allergenic, anti-atherogenic, anti-inflammatory, antimicrobial, antithrombotic and cardioprotective activities, and vasodilatory effects, which have been in part related to their antioxidant activity (Ferreira et al., 2009).

Overall, mannitol and trehalose were found in both samples, but fructose was only found in SP. Polyunsaturated fatty acids predominated over monounsaturated and saturated fatty acids. Linoleic, oleic and palmitic acids were abundant in both samples, but only SS gave considerable amounts of α-linolenic acid. The α-, γ- and δ-tocopherols were also quantified in both samples; γ- and δ-tocopherols were observed in higher levels in SS. Oxalic and fumaric acids were in both samples; malic acid was found in SP, while quinic and citric acids were observed in SS. Finally, protocatechuic and *p*-hydroxybenzoic acids were found in both samples, but *p*-coumaric acid was quantified in SP. It should be highlighted that this is the first report on fatty acids, organic acids, and phenolic compounds in *M. esculenta*.

3.2. Bioactive properties

3.2.1. Antioxidant activity

Antioxidant activity was accessed by five different assays that measured samples reducing power, free radicals scavenging activity and lipid peroxidation inhibition.

Both samples possessed antioxidant properties. SS gave higher reducing power measured by Ferricyanide/Prussian blue assay, and higher DPPH radical scavenging activity (lower EC_{50} values, **Table 3**). These results could be related to the higher total tocopherols and total organic acids content observed in SS (**Table 2**). Statistical correlations showed that, among the molecules present in the methanolic extracts, quinic and citric acids were the compounds that contribute more for DPPH scavenging activity (R^2=0.9971 and R^2=0.9972, respectively) and reducing power measured by Ferricyanide/Prussian blue assay (R^2=0.9991 and R^2=0.9988, respectively).

Otherwise, SP revealed higher lipid peroxidation inhibition evaluated by β-carotene/linoleate and TBARS assays, which seem to be more related with phenolic compounds, some individual organic acids namely malic and fumaric acids (higher content observed in this sample, **Table 2**), as also the reducing sugars fructose and mannitol (higher content observed in this sample, **Table 1**). In fact, protocatechuic (R^2=0.9938 and R^2=0.7892, respectively), *p*-hydroxybenzoic (R^2=0.9914 and R^2=0.7882, respectively) and *p*-coumaric (R^2=0.9528 and R^2=0.7503, respectively) acids proved to contribute more for the lipid peroxidation inhibition of the methanolic extracts, measured by both mentioned assays. Malic (R^2=0.9942 and R^2=0.7888, respectively) and fumaric (R^2=0.9917 and R^2=0.7853, respectively) acids, fructose (R^2=0.9929 and R^2=0.7887, respectively) and mannitol (R^2=0.9938 and R^2=0.7879, respectively) are also related to lipid peroxidation inhibition.

No significant differences were observed among reducing power measured by Folin-Ciocalteu assay. Sample of same species from Spain gave higher DPPH scavenging activity (Ramírez-Anguiano et al., 2007), but *M. esculenta* mycelium presented similar values of EC_{50} (Mau et al., 2004).

3.2.2. Antibacterial activity

Antibacterial activity was first tested using alternative disc-diffusion method to check if the extracts (2 mg/disc and 1 mg/disc) possessed any inhibitory effect on bacteria. Results for both samples of *M. esculenta* (SS and SP) showed almost the same values of inhibition zones (**Table 4**). The most sensitive species seemed to be *L. monocytogenes* treated with 2 mg of morel extracts per disc. The lowest inhibition zone was recorded for *E. coli* and was 6.31 mm and 6.44 mm for SP and SS, respectively. There were no inhibition zones on *S. typhimurium* and *E. coli* tested with 1 mg of extracts per disc. Commercial antibiotic streptomycin exhibited better antibacterial potential, with larger inhibition zones ranging from 6.12-18.13 mm.

Results of antibacterial activity towards pathogenic bacteria, evaluated by microdilution method, are also presented in **Table 4**. The extract of SS exhibited higher antibacterial activity than SP. *Staphylococcus aureus* was the most sensitive bacterial species for both morel extracts. The antibacterial activity of SP decreased in order: *S. aureus* > *L. monocytogenes* > *S. typhimurium* > *E. cloacae* > *E. coli.* Considering SS, the order was: *S. aureus* > *L. monocytogenes* > *S. typhimurium* > *E. coli* > *E. cloacae.* Comparing the results obtained for morel extracts with commercial antibiotics streptomycin and ampicillin, it is noticeable that SS exhibited stronger antimicrobial potential towards *S. aureus*, *S. typhimurium* and *L. monocytogenes* than antibiotics currently in use (streptomycin and ampicillin). This fact lead us to the conclusion that morel might be

alternative source of antimicrobial compounds. Nevertheless, further investigation on structure elucidation of active compounds from morel is necessary. Considering the differences among the results obtained by the two methods, antibacterial compounds showed low diffusion potential around the disc used in disc-diffusion method; these compounds (individually or in synergism) were much more active than commercial antibiotics in microdilution method probably due to direct contact with bacterial cells and better bioavailability. Moreover, the difference in antimicrobial activity observed for the two morel samples might be attributed to environment factors that might interfere in the synthesis of microbiologically active compounds. Tocopherols and organic acids contents were higher in the sample from Serbia (**Table 2**) and could be involved in its higher antimicrobial activity.

3.2.3. Demelanizing activity

In order to investigate the demelanizing activity of extracts, a suitable method was developed using the pathogenic strains of micromyetes. A major reason for studying demelanizing activity of extracts is the pigment's contribution to fungal virulence.

The results (**Table 5**) were expressed as minimum demelanizing concentrations (MDC), which were defined as sublethal and subinhibitory concentration necessary to provoke demelanization in fungus during 72 h. Both morel samples, from Portugal and Serbia, possessed demelanizing activity towards the studied pathogenic fungi. MDC values for SS were lower than the ones corresponding to SP. The most susceptible fungus was *P. ochrochloron*, while the most resistant one was *A. fumigatus*.

The green conidiophores of some *Aspergillus* and *Penicillium* species contains pigments belonging to the group of melanins: a green colored chromoprotein and a black insoluble pigment (Eisenman and Casadevall, 2012). Melanin production by fungi

contributes to the virulence of pathogens of humans as well as those of food crops (Rosa, Vieira, Santiago, & Rosa, 2010). Normal conidial pigment of *A. fumigatus* (**Figure 1a**) contains 1,8-dihydroxynaphthalene (DHN)-like pentaketide melanin. It was shown that this pigment has an important role in the protection of the fungus against immune effector cells; it is able to scavenge reactive oxygen species generated by alveolar macrophages and neutrophils of the host (Brakhage and Liebmann, 2005). Morphological changes in melanization of *A. fumigatus* are obvious from **Figure 1b** and showed complete depigmentation; samples were treated with *M. esculenta* extracts at MDC. Observing morphological changes of conidiphores it was determined that demelanized cultures of tested fungi interestingly possessed unusually small number of spores (**Figure 1d**) in comparison to those in untreated culture (**Figure 1c**). Melanin biosynthesis gene clusters have been characterized in several fungi including some *Aspergillus* and *Penicillium* species. In *A. fumigatus*, the cluster consists of six genes including alb1, the PKS gene, arp1, the scytalone reductase, arp2, the hydroxynaphthalene reductase, abr1, a multicopper oxidase, abr2, a putative laccase, and ayg1, a gene with unknown function (Tsai, Wheeler, Chang, & Kwon-Chung, 1999). The alb1 gene encodes a type I polyketide synthase. This gene was also found in some *Penicillium* species. It was shown that *A. fumigatus* mutants for this gene have white conidia while the normal conidia are greyish-green in color (Fujii et al., 2000). Thus, we may presume that the extracts of *M. esculenta* might directly be involved in the inhibition or modification of type I polyketide synthase, but further studies need to be conducted in order to check the mechanism of demelanization. The results for demelanizing activity are important, since MDC is sublethal to fungus being needed smaller doses of extract, in comparison to inhibitory and fungicidal doses.

4. Conclusion

Wild samples of *M. esculenta* are rich sources of carbohydrates (including free sugars) and proteins, and contain several bioactive compounds such as organic acids, phenolic compounds and tocopherols. Polyunsaturated fatty acids also predominated over mono and unsaturated fatty acids. SP gave higher radical scavenging activity and reducing power, while SS showed higher lipid peroxidation inhibition. Both samples, but mainly SS, gave antibacterial activity against five bacteria (in some cases even better than standard antibiotics) and demelanizing activity against four micromycetes (*Aspergillus fumigatus*, *Aspergillus flavus*, *Penicillium funiculosum* and *Penicillium ochrochloron*). As far as we know, this is the first study reporting chemical compounds and bioactivity of morel samples from Portugal and Serbia. Furthermore, a novel method for evaluation of demelanizing activity was presented. Further studies are needed in order to elucidate the mechanisms of action involved in morel bioactivity and contribution of the identified chemical compounds.

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Legends

Figure 1. (**a)** and (**b)** Cultures of *Aspergillus fumigatus* recorded under binocular magnifier; (**a)** Normal mycelium of *A. fumigatus* without treatment with *Morchella esculenta* extracts; (**b)** Demelanized mycelium of *A. fumigatus* treated with *M. esculenta* extracts. (**c)** and (**d)** Conidia of *A. flavus* recorded under light microscope; (**c)** Typical conidia of *A. flavus* with numerous spores; (**d)** Conidia of *A. flavus* with small amount of spores, treated with *M. esculenta* extracts.

Table 1. Proximate composition, free sugars and main fatty acids in *Morchella esculenta* fruiting bodies from Portugal and Serbia.

Parameter	SP	SS
Moisture (g/100 g fw)	90.79 ± 8.57	89.42 ± 2.50
Ash (g/100 g dw)	11.34 ± 0.81[a]	7.89 ± 0.12[b]
Carbohydrates (g/100 g dw)	74.55 ± 0.87[b]	78.36 ± 0.65[a]
Proteins (g/100 g dw)	11.52 ± 0.89[a]	11.49 ± 0.61[a]
Fat (g/100 g dw)	2.59 ± 0.42[a]	2.26 ± 0.10[a]
Energy (kcal/100 g dw)	367.59 ± 0.78[b]	379.76 ±0.01[a]
Fructose (g/100 g dw)	0.71 ± 0.03	nd
Mannitol (g/100 g dw)	11.54 ± 0.18[a]	1.08 ± 0.08[b]
Trehalose (g/100 g dw)	3.41 ± 0.01[b]	5.34 ± 0.17[a]
Total Sugars (g/100 g dw)	15.66 ± 0.13[a]	6.42 ± 0.25[b]
C16:0 (percent)	9.54 ± 0.04[b]	10.12 ± 0.10[a]
C18:0 (percent)	2.63 ± 0.01[a]	1.60 ± 0.03[b]
C18:1n9 (percent)	12.43 ± 0.26[a]	9.70 ± 0.04[b]
C18:2n6 (percent)	71.81 ± 0.16[a]	68.22 ± 0.00[b]
C18:3n3 (percent)	0.20 ± 0.05[b]	7.21 ± 0.08[a]
SFA (percent)	13.73 ±0.21[a]	13.14 ± 0.13[b]
MUFA (percent)	13.82 ±0.18[a]	10.92 ± 0.04[b]
PUFA (percent)	72.45 ±0 .03[b]	75.94 ± 0.08[a]

SP- sample from Portugal; SS- sample from Serbia; fw- fresh weight; dw- dry weight; nd- not detected. Palmitic acid (C16:0); Stearic acid (C18:0); Oleic acid (C18:1n9c); Linoleic acid (C18:2n6c); α-Linolenic acid (C18:3n3). SFA- saturated fatty acids; MUFA- monounsaturated fatty acids; PUFA- polyunsaturated fatty acids. The difference to 100% corresponds to other 18 less abundant fatty acids (data not shown). In each row, different letters mean significant differences between samples ($p<0.05$).

Table 2. Tocopherols, carotenoids, organic acids and phenolic compounds in *Morchella esculenta* fruiting bodies from Portugal and Serbia.

Compound	SP	SS
α-tocopherol	2.38 ± 0.00^{a}	2.40 ± 0.28^{a}
γ-tocopherol	12.41 ± 0.01^{b}	20.30 ± 0.71^{a}
δ- tocopherol	48.85 ± 4.48^{b}	98.60 ± 3.96^{a}
Total tocopherols (μg/100 g dw)	14.79 ± 0.01^{b}	121.30 ± 3.54^{a}
β-carotene	nd	nd
Lycopene (mg/100 g dw)	0.05 ± 0.00^{b}	0.27 ± 0.01^{a}
Oxalic acid	32.25 ± 2.68^{a}	32.73 ± 6.35^{a}
Quinic acid	nd	43.55 ± 0.24
Malic acid	199.10 ± 0.36	nd
Citric acid	nd	233.46 ± 4.49
Fumaric acid	47.81 ± 1.66^{a}	17.38 ± 0.38^{b}
Total organic acids (mg/100 g dw)	279.00 ± 4.24^{b}	327.00 ± 2.83^{a}
Protocatechuic acid	0.24 ± 0.01^{a}	0.06 ± 0.00^{b}
p-Hydroxybenzoic acid	0.10 ± 0.01^{a}	0.10 ± 0.01^{a}
p-Coumaric acid	0.01 ± 0.00	nd
Total phenolic compounds (mg/100 g dw)	0.35 ± 0.01^{a}	0.08 ± 0.00^{b}

SP- sample from Portugal; SS- sample from Serbia; dw- dry weight; nd- not detected. In each row, different letters mean significant differences between samples ($p<0.05$).

Table 3. Antioxidant activity of *Morchella esculenta* extracts from Portugal and Serbia.

Antioxidant activity	Assay	SP	SS
Reducing Power	Folin-Ciocalteu assay (mg GAE/g extract)	34.64 ±1.24[a]	32.17 ± 1.31[a]
	Ferricyanide/Prussian blue assay (EC_{50} value; mg/mL)	6.34 ± 0.07[a]	1.26 ± 0.12[b]
Scavenging activity	DPPH scavenging activity (EC_{50} value; mg/mL)	6.06 ± 0.05[a]	3.03 ± 0.11[b]
Lipid peroxidation inhibition	β-carotene/linoleate (EC_{50} value; mg/mL)	0.81 ± 0.02[b]	2.39 ± 0.09[a]
	TBARS assay (EC_{50} value; mg/mL)	1.01 ± 0.12[b]	2.23 ± 0.46[a]

SP- sample from Portugal; SS- sample from Serbia. Concerning the Folin-Ciocalteu assay, higher values mean higher reducing power; for the other assays, the results are presented in EC_{50} values, what means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance for the Ferricyanide/Prussian blue assay. Trolox (commercial standard) was used as positive control ($EC_{50} \leq 0.04$ mg/mL). In each row, different letters mean significant differences between samples ($p<0.05$).

Table 4. Antibacterial activity of *Morchella esculenta* extracts from Portugal and Serbia.

Disc-diffusion method (diameter of inhibition zones, mm)					
	SP		SS		Streptomycin
Bacteria	2 mg/disc	1 mg/disc	2 mg/disc	1 mg/disc	1 mg/disc
Staphylococcus aureus	7.22 ± 0.18	6.22 ± 0.27	7.34 ± 0.27	6.18 ± 0.06	6.12 ± 0.01
Salmonella typhimurium	6.48 ± 0.26	n.a.	6.47 ± 0.20	n.a.	14.14 ± 1.23
Listeria monocytogenes	7.80 ± 0.29	6.16 ± 0.04	8.34 ± 0.46	6.27 ± 0.13	18.13 ± 2.21
Escherichia coli	6.31 ± 0.23	n.a.	6.44 ± 0.10	n.a.	13.94 ± 0.69
Enteobacter cloacae	6.84 ± 0.61	6.23 ± 0.12	7.67 ± 0.65	7.05 ± 0.72	10.54 ± 0.61

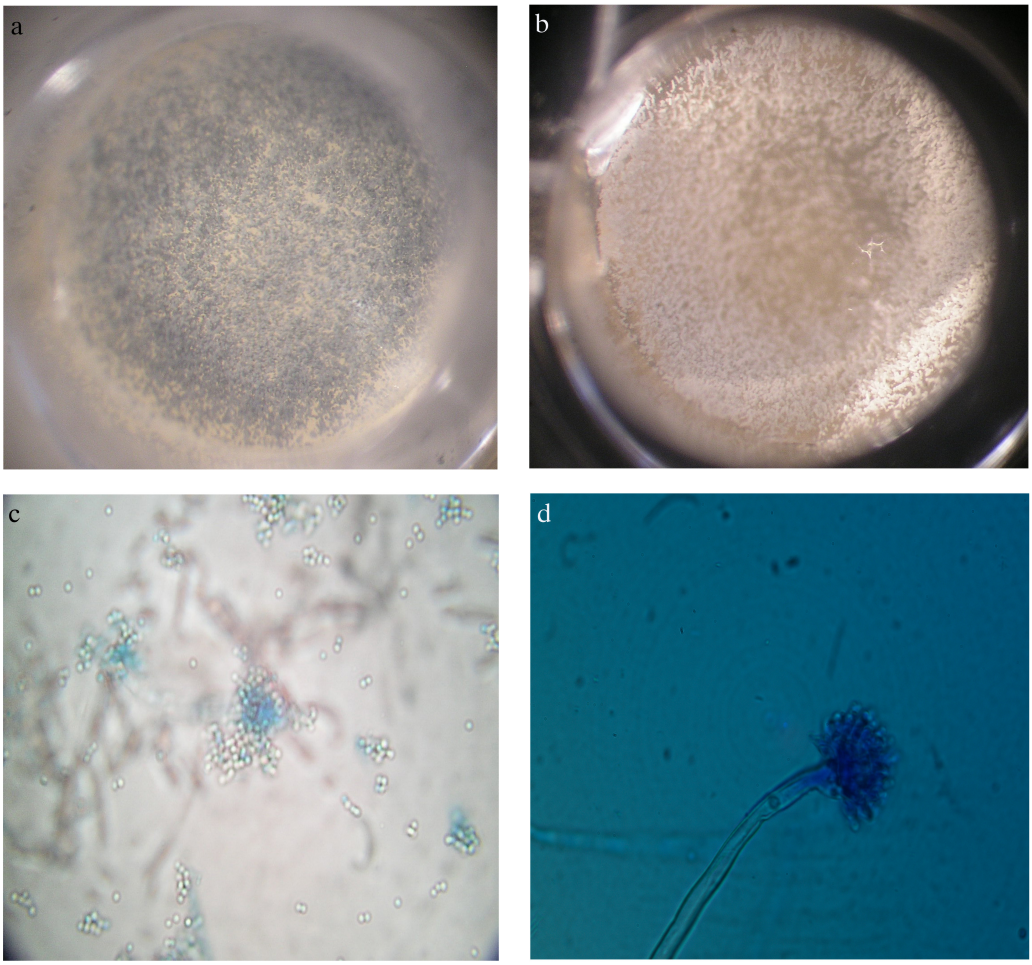
Microdillution method (MICs and MBCs, mg/mL)								
	SP		SS		Streptomycin		Ampicillin	
Bacteria	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Staphylococcus aureus	0.30 ± 0.06[a]	0.60 ± 0.04[a]	0.02 ± 0.00[b]	0.05 ± 0.00[b]	0.04 ± 0.00	0.09 ± 0.00	0.25 ± 0.05	0.37 ± 0.02
Salmonella typhimurium	2.50 ± 0.20[a]	5.00 ± 0.20[a]	0.10 ± 0.01[b]	0.30 ± 0.02[b]	0.17 ± 0.00	0.34 ± 0.01	0.37 ± 0.01	0.49 ± 0.03
Listeria monocytogenes	0.60 ± 0.04[a]	1.25 ± 0.03[a]	0.05 ± 0.00[b]	0.10 ± 0.01[b]	0.17 ± 0.01	0.34 ± 0.00	0.37 ± 0.01	0.49 ± 0.03
Escherichia coli	>10	>10	0.60 ± 0.02	1.25 ± 0.03	0.17 ± 0.03	0.34 ± 0.02	0.25 ± 0.05	0.49 ± 0.05
Enteobacter cloacae	5.00 ± 0.30[a]	5.00 ± 0.30[a]	1.25 ± 0.07[b]	1.25 ± 0.07[b]	0.26 ± 0.01	0.52 ± 0.02	0.37 ± 0.05	0.74 ± 0.07

SP- sample from Portugal; SS- sample from Serbia; n.a.- the extracts were not active at the tested concentrations; MIC- minimum inhibitory concentration; MBC- minimum bactericidal concentration. In each row different letters mean significant differences ($p<0.05$).

Table 5. Demelanizing activity of *Morchella esculenta* extracts from Portugal and Serbia.

Fungi	SP	SS
	MDC (mg/mL)	MDC (mg/mL)
Aspergillus fumigatus	0.60 ± 0.00^{a}	0.30 ± 0.00^{b}
Aspergillus flavus	0.05 ± 0.00^{a}	0.02 ± 0.00^{b}
Penicillium funiculosum	0.05 ± 0.00^{a}	0.02 ± 0.00^{b}
Penicillium ochrochloron	0.02 ± 0.00^{a}	0.01 ± 0.00^{b}

SP- sample from Portugal; SS- sample from Serbia; MDC- Minimum demelanizing concentration.



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