Polyporus squamosus (Huds.) Fr from different origins: Chemical characterization, screening of the bioactive properties and specific antimicrobial effects against *Pseudomonas aeruginosa*

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Abstract

Wild mushrooms are mainly collected during the rainy season and valued as a nutritious food and sources of natural medicines and nutraceuticals. The aim of this study was to determine the chemical composition and bioactive properties (antioxidant, antimicrobial and cytotoxicity) of *Polyporus squamosus* from two different origins, Portugal and Serbia. The sample from Portugal showed higher contents of as protein (17.14 g/100 g), fat (2.69 g/100 g), ash (3.15 g/100 g) and carbohydrates (77.02 g/100 g); the same sample gave the highest antioxidant activity: highest reducing power, DPPH radical scavenging activity, and lipid peroxidation inhibition in both β-carotene/linoleate and TBARS assay. These results could be related to its higher content in total tocopherols (1968.65 μg/100 g) and phenolic compounds (1.29 mg/100 g). Both extracts exhibited antibacterial activity against all the tested organisms. The samples from Serbia gave higher overall antibacterial activity and showed excellent antibiofilm activity (88.30 %). Overall, *P. squamosus* methanolic extracts possessed antioxidant, antimicrobial, antibiofilm and anti-quorum sensing activity, and without toxicity for liver cells. This investigation highlights alternatives to be explored for the treatment of bacterial infections, in particular against *Pseudomonas aeruginosa*. This study provides important results for the chemical and bioactive properties, especially antimicrobial activity of the mushroom *P. squamosus*. Moreover, to the authors' knowledge this is the first report on sugars, organic acids, and individual phenolic compounds in *P. squamosus*.

Keywords: *Polyporus squamosus*; Chemical characterization; Bioactive properties; Antimicrobial activity

1. Introduction

Fruiting bodies of mushrooms are appreciated, not only for texture and flavour but also for their chemical, nutritional (Barros, Cruz, Baptista, Estevinho & Ferreira, 2008) and bioactive properties including antioxidant (Ferreira, Barros & Abreu, 2009), antitumor (Ferreira, Vaz, Vasconcelos & Martins, 2010), antibacterial (Alves, Ferreira, Dias, Teixeira, Martins & Pintado, 2012), immunostimulatory, anti-fibrotic, anti-inflammatory (Taofiq et al., 2015), antiviral (Avtonomova & Krasnopolskaya, 2014), antifungal (Alves, Ferreira, Dias, Teixeira, Martins & Pintado, 2013), antiallergic, antiatherogenic, hypoglycaemic and hepatoprotective (Vaz, Heleno, Martins, Almeida, Vasconcelos & Ferreira, 2010) activities. In this sense, mushrooms have been recognized as functional foods, and a valuable source for the development of natural medicines and nutraceuticals (Heleno et al., 2013).

The antioxidant potential of mushrooms is mainly attributed to their composition in polysaccharides, phenolic compounds, tocopherols and organic acids (Elmastas, Isildak, Turkekul & Temur, 2007; Heleno et al., 2013). On the other hand, both low (*e.g.* sesquiterpenes, terpenes, steroids, anthraquinones, benzoic acid derivatives, quinolines, oxalic acid), and high (*e.g.* peptides and proteins) molecular weight compounds have been reported to play important role in mushrooms antimicrobial activity (Alves et al., 2012).

Some species of the genus *Polyporus* are rich in a variety of vitamins and minerals such as ascorbic acid, niacin, riboflavin, thiamine and some essential micronutrients (Okwulehie, Nwosu & Okoroafor, 2007). In particular, *Polyporus squamosus* (Huds.) Fr is an edible species at an early and tender stage, commonly used as a spice and being widely distributed in North America, Australia, Asia and Europe (Schmidt, 2006).

There are some reports on chemical composition of *P. squamosus* fruiting bodies (Dursun, Özcan, Kaşık & Öztürk 2006; Uzun, Gençcelep, Tunçtürk & Demırel, 2009; Akata, Ergonul & Kalyoncu, 2012; Ergönül, Akata, Kalyoncu & Ergönül, 2013), and its antioxidant (Elmastas *et al*. 2007; Akata *et al*. 2012; Dimitrijevic *et al*. 2015), immunomodulating (Babakhin, Logina, Nolte & DuBuske, 1996), immunosuppressive (Babakhin *et al*. 1999), antimicrobial and antiradical (Dimitrijevic *et al*. 2015) activities.

The present study aimed to provide more detailed information on chemical composition and bioactive properties (antioxidant, antimicrobial and cytotoxicity) of this species, by comparing samples from two different origins (Portugal and Serbia).

2. Materials and methods

2.1. Samples

Specimens of *Polyporus squamosus* (Huds.) Fr. were collected in Bragança (Northeast Portugal) and Jabučki rit (Northern Serbia) during April 2015 and 2012, respectively (in each case, ten fruiting bodies were collected). The authentications were performed 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 °C 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 Chemical Co. (St. Louis, MO, USA), as also standards of sugars, organic acids, phenolic acids, and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Tocol and tocopherols standards were purchased from Matreya (Plesant Gap, 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, Greenville, SC, USA). Mueller-Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia). Antibiotic standards were from Sigma and purchased from Galenika and Panfarma (Belgrade, Serbia).

2.3. Chemical composition

Nutritional value. The samples were analysed for chemical composition (moisture, proteins, fat, carbohydrates and ash) using the AOAC methods (AOAC 2005). 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°C. Total

carbohydrates were calculated by difference. Energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein + g carbohydrate) + 9 × (g fat).

Sugars. Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI, Knauer, Smartline system 1000; Berlin, Germany), after extraction and analysis procedures previously described by the authors (Heleno, Barros, Sousa, Martins, & Ferreira, 2009). The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard (melezitoze) method and sugar contents were further expressed in g per 100 g of dry weight (dw).

Fatty acids. Fatty acids were determined after a transesterification procedure as described previously by the authors (Heleno *et al.* 2009). The analysis was carried out in a gas–liquid chromatography (GC DANI 1000; Contone, Switzerland) with flame ionization detection (FID at 260 °C), using a capillary column (50% cyanopropyl-methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 μm df, Macherey–Nagel, Düren, Germany), with a flow-rate of 4.0 mL/min (0.61 bar), measured at 50 °C and hydrogen as carrier gas. Split injection (1:40) was carried out at 250 °C. Fatty acid identification was performed by comparing the relative retention times of FAME peaks from samples with standards. The results were expressed as relative percentage of each fatty acid.

Tocopherols. Tocopherols were determined following a procedure previously described by the authors (Heleno, Barros, Sousa, Martins & Ferreira, 2010) using HPLC-fluorescence (Knauer, Smartline system 1000, FP-2020; Jasco, Easton, MD, USA). Quantification was based on the

fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in µg per 100 g of dry weight (dw).

Organic acids. Organic acids were determined by ultrafast liquid chromatography coupled to a photodiode array detector (UFLC-PAD, Shimadzu Coperation, Kyoto, Japan), following a procedure previously described by the authors (Barros, Pereira, & Ferreira, 2013). The organic acids found 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 (dw).

Phenolic compounds. Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA) as previously described by the authors (Heleno, Barros, Martins, Queiroz, Santos-Buelga, & Ferreira 2012). Double online detection was carried out in the diode array detector (DAD) using 280 nm and 370 nm and in a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany). 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 mg per 100 g of dry weight.

2.4. Preparation of the extracts for evaluation of bioactive properties

Extract preparation. Each methanolic extract was obtained from the lyophilized mushroom material. The sample material (~1.5 g) was extracted by stirring with 30 mL of methanol (25 ºC

at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with an additional 30 mL of methanol (25 ºC at 150 rpm) for 1 h. The combined methanolic extracts were evaporated at 40 ºC (rotary evaporator Büchi R-210, Flawil, Switzerland) to dryness. The final extracts were redissolved in i) methanol for antioxidant activity assays or ii) 5% DMSO containing 0.02% Tween 80 for antimicrobial activity and cytotoxicity assays.

Antioxidant activity. Successive dilutions of the stock solution were made and submitted to *in vitro* assays already described by the authors (Heleno *et al*. 2010) to evaluate the antioxidant activity of the samples. The sample concentrations providing 50% of antioxidant activity (EC_{50}) or 0.5 of absorbance ($EC_{0.5}$) were respectively calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene/linoleate and TBARS assays) or absorbance at 690 nm (reducing power assay) against extract concentrations. The commercial standard trolox was used as positive control.

DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution (Heleno et al., 2010). Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above (Heleno *et al.* 2010). Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2h of assay/initial absorbance) × 100 (Heleno *et al.*

2010). Lipid peroxidation inhibition in porcine brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: [(A - B)/A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively (Heleno *et al.* 2010).

Antimicrobial properties. For the antifungal bioassays, the following microfungi were used: *Aspergillus fumigatus* (1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112), *Penicillium aurantiogriseum* (food isolate) and *Trichoderma viride* (IAM 5061). For the antibacterial bioassays, Gram-negative bacteria were used: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030) and the following Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković´", Belgrade, Serbia.

In order to investigate the antimicrobial activity of the *P. squamosus* methanolic extract, a modified microdilution technique was used (Espinel-Ingroff, 2001). Bacterial species were cultured overnight at 37 °C in Luria broth medium. The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The bacterial cells and fungal spore suspension was adjusted with sterile saline to a concentration of

approximately 1.0×10^5 in a final volume of 100 µL per well. The inocula were stored at 4 °C for further use. Dilutions of the inocula were cultured on Müller-Hinton agar for bacteria and solid Malt Agar for fungi to verify the absence of contamination and to check the validity of the inoculum.

Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microtiter plates. The extracts investigated were dissolved in 5% DMSO and added in broth medium (bacteria)/broth malt medium (fungi) with inocula. The microplates were incubated for 48 h at 37 °C for bacteria or 72 h at 28 °C for fungi. The following day, 30 µL of 0.2 mg/mL solution of INT (*p*-iodonitrotetrazolium violet) was added, and the plates were returned to the incubator for at least one-half hour to ensure adequate color reaction. Inhibition of growth was indicated by a clear solution or a definite decrease in color reaction. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs (CLSI, 2009; Tsukatani *et al*. 2012). The minimum bactericidal concentrations (MBCs) and minimum fungicidal concentrations (MFCs) were determined by serial subcultivation of a 2 μL sample into microtiter plates containing 100 μL of broth per well and further incubation for 48 h at 37 °C or 72 h at 28 °C. The lowest concentration with no visible growth was defined as MBC/MFC, respectively, indicating 99.5% killing of the original inoculum. Streptomycin and ampicillin were used as standards in the antibacterial assays, while bifonazole and ketokonazole were used as standards in the antifungal susceptibility tests. 5% DMSO was used as a negative control.

Inhibition of biofilm formation in Pseudomonas aeruginosa. *Pseudomonas aeruginosa* PA01 (ATCC 27853) was from the collection of the Mycoteca, Institute for Biological Research

"Sinisa Stankovic", Belgrade, Serbia. Bacteria were routinely grown in Luria-Bertani (LB) medium (1% w/v NaCl, 1% w/v tryptone, 0.5% w/v yeast extract) with shaking (220 rpm) and cultured at 37°C. The effect of different concentrations of extract (0.5 to 0.125 of MIC;) on biofilm forming ability was tested on polystyrene flat-bottomed microtitre 96 well plates as described by (Drenkard & Ausubel, 2002) with some modifications. Briefly, 100 µL of overnight culture of *P. aeruginosa* (inoculum size was 1×10^7 CFU/mL) was added to each well of the plates in the presence of 100 µL subinhibitory concentrations (subMIC) of extract (0.5, and 0.125 MIC) or 100 mL medium (control). After incubation for 24 h at 37 °C, each well was washed twice with sterile PBS (pH 7.4), dried, stained for 10 min with 0.1% crystal violet in order to determine the biofilm mass. After drying, 200 µL of 95% ethanol (v/v) was added to solubilize the dye that had stained the biofilm cells. The excess stain was washed off with dH_2O. After 10 min, the content of the wells was homogenized and the absorbance at $\lambda = 625$ nm was read on a Sunrise™- Tecan ELISA reader (Mannedorf, Switzerland).

Inhibition of twitching and flagella motility of Pseudomonas aeruginosa. After growth in the presence or absence of the extracts (subMIC), streptomycin and ampicillin (subMIC), the cells of *P. aeruginosa* PA01 were washed twice with sterile PBS and resuspended in PBS at 1×10^8 cfu/mL (OD of 0.1 at 660 nm). Briefly, cells were stabbed into a nutrient agar plate with a sterile toothpick and incubated overnight at 37°C. Plates were then removed from the incubator and incubated at room temperature for two more days. Colony edges and the zone of motility were measured with a light microscope (O'Toole & Kolter, 1998a, b). Fifty microlitres of the extract was mixed into 10 mL of molten MH (Mueller-Hinton) agar medium and poured immediately over the surface of a solidified LB agar plate as an overlay. The plate was point inoculated with

an overnight culture of PAO1 once the overlaid agar had solidified and incubated at 37 °C for 3 days. The extent of swimming was determined by measuring the area of the colony (O'May & Tufenkji, 2011). The colony diameters were measured three times in different direction.

Inhibition of PA01 pyocyanin production. Overnight culture of *P. aeruginosa* PA01 was diluted to $OD_{600\ nm}$ 0.2. Then, the extract 0.5 MIC was added to *P. aeruginosa* (4.75 mL) and incubated at 37 °C for 24 h. The treated culture was extracted with chloroform (3 mL), followed by mixing the chloroform layer with 0.2 M HCl (1 mL). Absorbance of the extracted organic layer was measured at 520 nm using a Shimadzu UV1601 spectrophotometer (Kyoto, Japan) (Sandy & Foong-Yee, 2012).

Cytotoxicity in liver primary cell culture. A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, according to a procedure established by the authors (Guimarães *et al*. 2013); it was designed as PLP2. The growth inhibition was evaluated by the sulphorhodamine (SRB) assay. The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

2.5. Statistical analysis

Ten specimens of SP and ten specimens of SS were used to obtain both samples. All the extractions were performed in triplicate as also each one of the analyses. The results are expressed as mean values and standard deviation (SD). The results were analyzed using a Student´s *t*-test with $\alpha = 0.05$, to determine the significant difference among the two different samples; this analysis was performed for the chemical composition and antioxidant activity. For

the remaining results (Tables 4-7), the analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. These treatments were carried out using SPSS v. 22.0 program.

3. Results and discussion

3.1. Chemical composition

The results of the chemical composition (nutritional value, primary and secondary metabolites) of *P. squamosus* fruiting bodies from Portugal (SP) and Serbia (SS) are shown in **Tables 1** and **2**. Carbohydrates were the most abundant macronutrients, followed by proteins and ash. Fat content was low and similar in both samples. However, the energy value of SP was slightly higher than the one obtained for SS (**Table 1**). The nutritional composition of SP was similar to a specimen from Anatolia (Turkey): protein 13.32%, fat 3.98%, ash 7.14% and carbohydrates 65.24% (Akata *et al*. 2012); Nevertheless, Uzun et al. (2009) described an extremely high content of proteins (64.70%) in a sample of *P. squamosus* from Turkey.

Rhamnose, fructose, mannitol and trehalose were found in both samples (**Table 1**). The SS gave the highest levels of total sugars, while revealed the highest levels of trehalose and mannitol. 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 (C18:2n6), oleic (C18:1n9) or palmitic (C16:0) acids, depending on the sample. Curiously, the stearic acid (C18:0) was quantified in higher amounts in SP, being similar to the concentration reported in a sample from Ankara (Turkey) (Ergönül *et al*. 2013).

Regarding tocopherols, α-, β and γ-tocopherols were found in SS, but in SP the γ-tocopherol was not identified. SP contained higher total tocopherols content than SS, and revealed a higher level of β-tocopherol; γ-tocopherol predominated in SS (**Table 2**). However, in a sample from Turkey, only α-tocopherol was detected (Elmastas *et al*. 2007). These differences could be related to natural variability inherent to samples grown under different environmental conditions. Tocopherols are important fat-soluble antioxidants, acting in the cellular membrane; due to their role as scavengers of free radicals, these molecules act to protect human cells against degenerative malfunctions (Lobo, Patil, Phatak, & Chandra, 2010).

Among organic acids, it was possible to quantify oxalic, malic and fumaric acids in both samples. The oxalic acid might have toxicity effects (Nagarajkumar, Jayaraj, Muthukrishnan, Bhaskaran & Velazhahan, 2005), while fumaric acid possesses interesting biological effects such as anti-inflammatory, neuroprotective, chemo preventive and antimicrobial activity (Baati, Horcajada, Gref, Couvreur & Serre, 2011). Malic acid was found in high amounts in SS (**Table 2**). It should be highlighted that the content of these compounds in food not only influences their flavor, but also their stability, nutrition, and acceptability (Begić-Akagić *et al*. 2014).

p-Hydroxybenzoic acid and cinnamic acid were found in both samples (**Table 2**); the first one predominates in SP, while cinnamic acid was more abundant in SS. Elmastas et al. (2007) described that the total concentration of phenolic compounds in methanolic extracts of *P. squamosus* from Turkey was 13.9 mg/g. Phenolic compounds contribute directly to the antioxidative action and play an important role in stabilizing lipid peroxidation (Elmastas *et al*. 2007); these molecules exhibit a wide range of physiological properties such as anti-allergenic, anti-atherogenic, anti-inflammatory, antimicrobial, antithrombotic and cardio protective

activities, and vasodilator effects, which have been in part related to their antioxidant activity (Ferreira *et al.* 2009).

To the authors knowledge this is the first report on sugars, organic acids, and individual phenolic compounds in *P. squamosus*. The differences registered in the other chemical compounds between SP, SS and the previously mentioned Turkish samples could be related with different growth conditions including the soil and climate, but also with collection and storage procedures (Elmastas *et al.* 2007; Akata *et al.* 2012; Dimitrijevic *et al.* 2015).

3.2. Bioactive properties

3.2.1. Antioxidant activity

The antioxidant activity of *P. squamosus* methanolic extracts was assessed by four different assays that measured samples reducing power, free radicals scavenging activity and lipid peroxidation inhibition (**Table 3**).

Both samples possessed antioxidant properties; even though these results are much higher than the positive control trolox (which is a single molecule) they are comparable and in the same range as other mushrooms. SP extract gave higher reducing power, DPPH radical scavenging activity, and lipid peroxidation inhibition in both β-carotene/linoleate and TBARS assay (**Table 3**). These results could be related to its higher content in total tocopherols and phenolic compounds (**Table 2**). Other authors have previously reported higher DPPH scavenging activity and reducing power in *P. squamosus* from Turkey (Elmastas *et al*. 2007; Akata *et al*. 2012).

Locations generally affect the composition of wild mushrooms, which has reflects on their bioactive properties. These differences might be due related with growth conditions, genetic

factors, geographical variations and analytical procedures (Dursun, Özcan, Kaşık & Öztürk 2006; Sadiq, Bhatti & Hanif, 2008; Munkhgerell *et al*. 2013).

3.2.2. Antimicrobial activity

The results of the antifungal activity are similar for both extracts. Antifungal drugs ketoconazole and bifonazole exhibited high activity in the range of 0.10-3.50 mg/mL (**Table 4**). However, the effect of ketoconazole is lower than both of the extracts in the case of *P. funiculosum*, a very well-known plant pathogen, which might has future prospects. Also, it is important to notice that both extracts showed inhibitory activity against all the tested micromycetes. In the last decade, a number of studies were conducted to investigate the possible antimicrobial activity of mushrooms.

Both extracts exhibited good antibacterial activity against all the tested organisms. In general, SS showed higher overall antibacterial activity than SP. Nevertheless, this extract exhibited excellent activity against *S. aureus* with MIC value of 0.20 mg/mL and MBC value of only 0.39 mg/mL (**Table 5**). Commercial antibiotics streptomycin and ampicillin showed higher activity against all the tested bacteria. Still, all the side-effects of these clearly efficient antimicrobial drugs, the existence of other sources of effective antimicrobial agents, which can also be used as food with no side effects or whatsoever, draws attention. *B. cereus* proved to be the most susceptible to the activity of the methanolic extract from SS. Other species (namely, *L. monocytogenes*, *M. flavus*, *P. aeruginosa*) showed rather uniform sensitivity to the extracts (**Table 5**).

In a recent study, Dimitrijevic et al. (2015) described the antimicrobial activity of a *P. squamosus* extract soluble in 100.0% DMSO, against a panel of pathogenic bacterial strains,

highlighting its activity against *S. aureus* (MIC/MBC = 6.3/6.3 mg/mL). Since weak antimicrobial activity is demonstrated in this work, antimicrobial activity of *P. squamosus* could be related to the group of compounds previously identified in mushrooms. It is known that the antimicrobial activity of mushrooms can be attributed to several compounds such as sesquiterpenes and other terpenes, steroids, anthraquinones, benzoic acid derivatives, and quinolines, oxalic acid as well as high molecular weight compounds (peptides and proteins) (Alves *et al.* 2012).

3.2.3. Specific effects against Pseudomonas aeruginosa

Numerous studies indicate that bacteria are far more resistant (up to 1000 times) to antibiotics when in form of biofilm than when in free form (Potera, 2010). Therefore, a new approach for breaking down this defence system is needed. Chemical analyses of extracts derived from natural sources point to the fact that they are a mixture of chemical and biological active substances, which multiply their potential to disrupt this formation in a mechanism that is still unclear. The effect of the two *Polyporus* extracts on biofilm formation and other quorum-sensing regulated functions were tested at subinhibitory level (0.5 and 0.125 MIC values) (**Table 6**). SS showed excellent antibiofilm activity at the aforementioned subinhibitory levels (88.30 % and 84.30 %, respectively) while SP did not exhibit antibiofilm activity. In the case of SS, the results showed higher antibiofilm-forming activity than ampicillin and streptomycin (**Table 6**). Twitching activity of the two tested extracts was also higher in SS, in terms of PAO1 colony diameter, color and edge (**Table 7**). Regarding pyocyanin production, results are not consistent with previously mentioned quorum-sensing regulated functions. SS showed lower reduction of pigment production than SP. As it can be seen in **Figure 1**, PAO1 produced significant amount of

pyocyanin (83.12%), while the production was reduced to a level of 44.5% in the presence of SP extract. Positive controls (streptomycin and ampicillin) showed lower reduction of pigment production (**Figure 1**). Nevertheless, despite the fact that several mechanisms of action have been proposed as anti-QS interfering signals, no specific mechanism has been suggested with certainty.

3.2.4. Cytotoxicity

An initial assessment of possible extracts toxicity was performed by studying their *in vitro* cell growth inhibition activity in a porcine liver primary cell culture (PLP2) established in our laboratory. Mammalian hepatocytes represent an obligatory step in the evaluation of toxic compounds that lead to the production of various metabolites, which are the ultimate cause of toxicity. Porcine liver as an *in vitro* cytotoxicity model is acceptable because it is very similar to human in terms of cellular and physiological functioning. Furthermore, it is faster and cheaper than using human primary cell cultures or even fresh isolated human hepatocytes, and avoids some ethical concerns (Bendixen, Danielsen, Larsen, & Bendixen, 2010; Abreu et al., 2011). The tested extracts did not show any toxicity for the liver primary cell culture PLP2, up to the maximal concentration tested (no significant inhibition of the cells growth was observed in the presence of the extracts), while the positive control ellipticine gave a GI_{50}=2.06 ± 0.03 µg/mL.

Overall, the present study proves that mushroom samples from different origins have dissimilar chemical composition and, therefore, different bioactivities, which is in agreement with previous studies (Vieira *et al*. 2014). *P. squamosus* methanolic extracts possessed antioxidant, antimicrobial, antibiofilm and anti-quorum sensing activity, and without toxicity for liver cells.

In light of new research, this may be the corner stone for alternative strategy for the treatment of bacterial infections, in particular against *Pseudomonas aeruginosa*.

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

	SP	SS	*t*-Students test *p*-value
Nutritional value			
Moisture (g/100 g fw)	82.81±1.32	85.21±0.98	0.012
Ash (g/100 g dw)	3.15±0.06	8.42±0.07	<0.001
Carbohydrates (g/100 g dw)	77.02±0.11	78.36±0.66	0.025
Proteins (g/100 g dw)	17.14±0.01	10.65±0.40	<0.001
Fat (g/100 g dw)	2.69±0.09	2.86±0.28	0.239
Energy (kcal/100 g dw)	400.88±0.13	380.62±1.20	0.001
Free sugars			
Rhamnose (g/100 g dw)	0.16±0.01	0.34±0.01	<0.001
Fructose (g/100 g dw)	1.41±0.09	0.62±0.02	<0.001
Mannitol (g/100 g dw)	0.16±0.01	2.48±0.24	<0.001
Trehalose (g/100 g dw)	5.25±0.18	7.13±0.12	<0.001
Total Sugars (g/100 g dw)	6.98±0.28	10.57±0.33	<0.001
Fatty acids			
C16:0 (percent)	19.80±0.30	15.54±0.22	<0.001
C18:0 (percent)	11.66±0.14	1.62±0.01	<0.001
C18:1n9 (percent)	17.17±1.51	24.64±0.07	<0.001
C18:2n6 (percent)	45.17±1.06	52.80±0.16	<0.001
SFA (percent)	34.11±0.53	20.69±0.23	0.001
MUFA (percent)	19.77±1.59	25.97±0.05	<0.001
PUFA (percent)	46.12±1.06	53.34±0.17	<0.001

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). SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids. The difference to 100% corresponds to other 23 less abundant fatty acids (data not shown).

Table 2. Tocopherols, organic acids and phenolic compounds in *Polyporus squamosus* fruiting bodies from Portugal and Serbia.

	SP	SS	*t*-Students test *p*-value
Tocopherols			
α-tocopherol	4.06±0.71	11.25±1.11	<0.001
β-tocopherol	1964.59±62.70	17.17±0.27	<0.001
γ-tocopherol	nd	89.23±14.76	-
Total tocopherols (μg/100 g dw)	1968.65±63.42	117.66±16.14	<0.001
Organic acids			
Oxalic acid	0.08±0.01	0.45±0.06	<0.001
Malic acid	1.33±0.01	3.96±0.17	<0.001
Fumaric acid	0.21±0.01	0.41±0.01	<0.001
Total organic acids (g/100 g dw)	1.62±0.01	4.82±0.11	<0.001
Phenolic and related compounds			
p-Hydroxybenzoic acid (mg/100 g dw)	1.29±0.01	0.63±0.03	<0.001
Cinnamic acid (mg/100 g dw)	0.04±0.01	0.13±0.01	<0.001

SP - sample from Portugal; SS - sample from Serbia; dw - dry weight; nd - not detected.

Table 3. Antioxidant activity of methanolic extracts of *Polyporus squamosus* fruiting bodies from Portugal and Serbia.

Antioxidant activity	Assay	SP	SS	*t*-Students test *p*-value
Reducing power	Ferricyanide/Prussian blue assay ($EC_{0.5}$ value; mg/mL)	1.27±0.07	3.53±0.03	<0.001
Scavenging activity	DPPH scavenging activity (EC_{50} value; mg/mL)	8.30±0.12	13.57±0.19	<0.001
Lipid peroxidation inhibition	β-carotene/linoleate assay (EC_{50} value; mg/mL)	3.60±0.05	8.41±0.11	<0.001
	TBARS assay (EC_{50} value; mg/mL)	0.86±0.02	2.03±0.01	<0.001

SP - sample from Portugal; SS - sample from Serbia. The results are presented in $EC_{0.5}$/EC_{50} values which means that higher values correspond to lower reducing power/antioxidant potential. $EC_{0.5}$: Extract concentration corresponding to 0.5 of absorbance in the Ferricyanide/Prussian blue assay. EC_{50}: Extract concentration corresponding to 50% of antioxidant activity in DPPH scavenging activity and lipid peroxidation inhibition assays. Trolox EC_{50} values: 0.04 mg/mL (reducing power), 0.04 mg/mL (DPPH scavenging activity), 0.02 mg/mL (β-carotene bleaching inhibition) and 0.02 mg/mL (TBARS inhibition).

Table 4. Antifungal activity of methanolic extracts (mg/mL) of *Polyporus squamosus* fruiting bodies from Portugal and Serbia.

	SS	SP	Ketoconazole	Bifonazole
Bacteria	MIC MFC	MIC MFC	MIC MFC	MIC MFC
A. fumigatus	0.75±0.02^{c}	3.13±0.02^{d}	0.20±0.01^{b}	0.15±0.02^{a}
	1.50±0.01^{c}	6.25±0.02^{d}	0.50±0.01^{b}	0.20±0.01^{a}
A. versicolor	0.40±0.03^{c}	0.78±0.01^{d}	0.20±0.02^{b}	0.10±0.01^{a}
	0.75±0.02^{c}	1.56±0.02^{d}	0.50±0.01^{b}	0.20±0.02^{a}
A. ochraceus	0.40±0.01^{b}	1.56±0.01^{c}	0.15±0.01^{a}	0.15±0.02^{a}
	0.75±0.02^{b}	3.13±0.03^{c}	0.20±0.02^{a}	0.20±0.03^{a}
A. niger	0.75±0.03^{c}	1.56±0.01^{d}	0.20±0.01^{b}	0.15±0.01^{a}
	1.50±0.02^{c}	3.13±0.02^{d}	0.50±0.02^{b}	0.20±0.02^{a}
T. viride	0.40±0.01^{c}	1.56±0.03^{d}	0.20±0.03^{b}	0.10±0.02^{a}
	0.75±0.01^{c}	3.13±0.03^{d}	0.30±0.02^{b}	0.20±0.01^{a}
P. funiculosum	0.75±0.02^{b}	1.56±0.01^{c}	2.50±0.01^{d}	0.20±0.03^{a}
	1.50±0.01^{b}	3.13±0.02^{c}	3.50±0.02^{d}	0.25±0.01^{a}
P. ochrochloron	0.75±0.02^{b}	0.78±0.01^{b}	0.20±0.01^{a}	0.20±0.02^{a}
	1.50±0.03^{c}	1.56±0.03^{c}	0.50±0.02^{b}	0.25±0.01^{a}
P. verucosum	1.50±0.01^{c}	0.78±0.02^{b}	0.25±0.02^{a}	0.25±0.02^{a}
	3.00±0.02^{c}	1.56±0.01^{b}	0.50±0.02^{a}	0.50±0.03^{a}

SP - sample from Portugal; SS - sample from Serbia; MIC - Minimum inhibitory concentrations; MFC - minimum fungicidal concentrations. In each line different letters mean significant differences between species (p<0.05).

Table 5. Antibacterial activity of methanolic extracts (mg/mL) of *Polyporus squamosus* fruiting bodies from Portugal and Serbia.

Bacteria	SS	SP	Ampicillin	Streptomycin
	MIC	MIC	MIC	MIC
	MBC	MBC	MBC	MBC
S. aureus	1.50 ± 0.01^{c}	0.20 ± 0.03^{b}	0.10 ± 0.03^{a}	0.25 ± 0.03^{bc}
	3.00 ± 0.02^{d}	0.39 ± 0.02^{b}	0.15 ± 0.01^{a}	0.5 ± 0.01^{c}
B. cereus	0.40 ± 0.03^{c}	1.56 ± 0.02^{d}	0.10 ± 0.01^{b}	0.05 ± 0.02^{a}
	0.75 ± 0.01^{c}	3.13 ± 0.01^{d}	0.15 ± 0.02^{b}	0.10 ± 0.03^{a}
L. monocytogenes	1.50 ± 0.03^{b}	3.13 ± 0.03^{c}	0.15 ± 0.03^{a}	0.15 ± 0.03^{a}
	3.00 ± 0.01^{b}	6.25 ± 0.02^{c}	0.30 ± 0.03^{a}	0.30 ± 0.01^{a}
M. flavus	1.50 ± 0.01^{b}	3.13 ± 0.01^{c}	0.10 ± 0.03^{a}	0.125 ± 0.02^{a}
	3.00 ± 0.02^{c}	6.25 ± 0.03^{d}	0.15 ± 0.02^{a}	0.25 ± 0.01^{b}
P. aeruginosa	1.50 ± 0.02^{c}	3.13 ± 0.02^{d}	0.10 ± 0.01^{b}	0.05 ± 0.03^{a}
	3.00 ± 0.01^{c}	6.25 ± 0.02^{d}	0.20 ± 0.03^{b}	0.10 ± 0.03^{a}
E.coli	0.56 ± 0.03^{c}	3.13 ± 0.02^{d}	0.30 ± 0.02^{b}	0.05 ± 0.01^{a}
	1.14 ± 0.03^{c}	6.25 ± 0.02^{d}	0.50 ± 0.03^{b}	0.10 ± 0.01^{a}
S. typhimurium	0.75 ± 0.01^{d}	0.39 ± 0.01^{c}	0.15 ± 0.01^{b}	0.05 ± 0.02^{a}
	1.50 ± 0.03^{d}	0.78 ± 0.02^{c}	0.20 ± 0.03^{b}	0.10 ± 0.02^{a}
En. cloacae	0.40 ± 0.01^{c}	1.56 ± 0.02^{d}	0.15 ± 0.01^{b}	0.05 ± 0.03^{a}
	1.50 ± 0.01^{c}	3.13 ± 0.03^{d}	0.20 ± 0.01^{b}	0.10 ± 0.02^{a}

SP - sample from Portugal; SS - sample from Serbia; MIC - Minimum inhibitory concentrations; MBC - minimum bactericidal concentrations. In each line different letters mean significant differences between species ($p<0.05$).

Table 6. Effects of *Polyporus squamosus* methanolic extracts on biofilm formation (%*) of *P. aeruginosa*.

Agents	SS	SP	Ampicillin	Streptomycin
0.5MIC	88.30±0.65[a]	-	69.16±0.65[b]	49.40±0.46[c]
0.125MIC	84.30±0.55[c]	-	92.16±0.37[a]	88.36±0.42[b]

SP - sample from Portugal; SS - sample from Serbia; *Biofilm formation values were calculated as: (mean A_{620} treated well)/(mean A_{620} control well) x 100. In each line different letters mean significant differences between species ($p<0.05$).

Table 7. Twitching activity of *Polyporus squamous* methanolic extract.

Agents	SS	SP	Streptomycin	Ampicillin	Control P. a. 10^9
Colony diameter (mm ± SD)	7.67 ± 0.43^{a}	18.67 ± 0.22^{c}	8.33 ± 0.15^{b}	20.00 ± 0.35^{cd}	25.00 ± 0.25^{d}
Colony color	white	pale green	white	pale green	green
Colony edge	reduced flagella	no flagella	flat	reduced flagella	regular flagella

SP - sample from Portugal; SS - sample from Serbia. In the first line different letters mean significant differences between species ($p<0.05$).

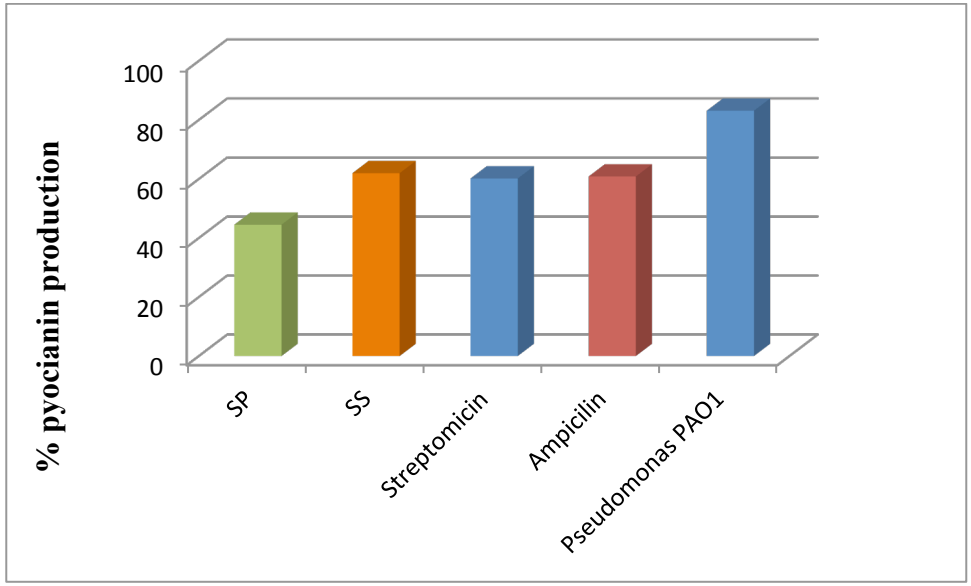

Figure 1. Reduction of pyocianin production.