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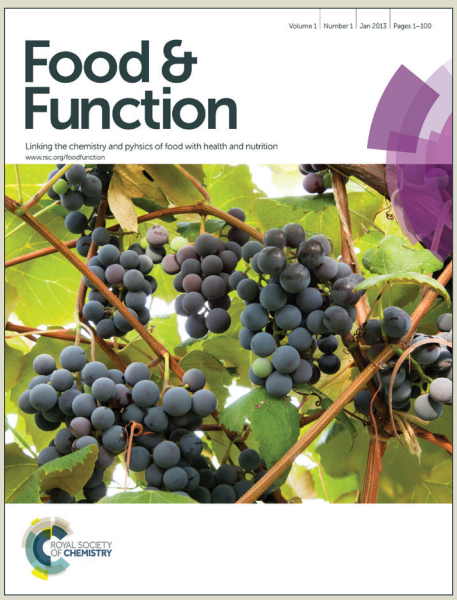

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Study on chemical, bioactive and food preserving properties of *Laetiporus sulphureus* (Bull.: Fr.) Murr

Jovana Petrović[a], Dejan Stojković[a], Filipa S. Reis[b], Lillian Barros[b], Jasmina Glamočlija[a], Ana Ćirić[a], Isabel C.F.R. Ferreira[b,*], Marina Soković[a,*]

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

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

Running title: Chemistry and bioactivities of *L. sulphureus*

* Authors to whom correspondence should be addressed (Isabel C.F.R. Ferreira; e-mail: iferreira@ipb.pt; telephone +351-273-303219; fax +351-273-325405 and Marina D. Soković; e-mail: mris@ibiss.bg.ac.rs; telephone +381-11-2078419; fax +381-11-2761433).

Abstract

Laetiporus sulphureus (Bull.: Fr.) Murr. was studied regarding the nutritional value, bioactive compounds, *in vitro* antioxidant, antimicrobial and antitumor activities. The studied mushroom is a rich source of carbohydrates and proteins. Mannitol and trehalose were the main free sugars, and polyunsaturated fatty acids. α-, γ- and δ-Tocopherols were found. Oxalic and citric acids were the most abundant organic acids; cinnamic and *p*-hydroxybenzoic acids were quantified in the methanolic extract and could be related to the antioxidant properties. It was the polysaccharidic extract that exhibited, higher antioxidant and antimicrobial activities, indicating that compounds present in this extract possess stronger bioactivity. Only polysaccharidic extract revealed antiproliferative activity of human tumor cell lines. A suitable model system with chicken pate was also developed to test antimicrobial preserving properties of *L. sulphureus*. The methanolic extract was used to examine *in situ* preserving properties against *Aspergillus flavus* and demonstrated excellent preserving potential.

Keywords: *Laetiporus sulphureus*; chemical characterization; antioxidant; antimicrobial; antitumour; preserving chicken pate.

Introduction

Mushrooms are consumed in many countries as a culinary delicacy, especially for their taste.[1] In recent years, scientists have expanded their research area concerning other uses of mushrooms namely, medicinal and food preserving purposes.

Regarding medicinal value, wild mushrooms have emerged as an important source of antioxidant compounds, which is very important since it has been recommended the intake of foods rich in compounds that could eliminate free radicals. These reactive species are produced as a part of the normal process of aerobic metabolism, but they can cause structural damage to cells, being implicated in several chronic diseases such as various types of cancer, cardiovascular diseases or diabetes.[2,3] Particularly, cancer is amongst the most common diseases among people. According to World Health Organization, more than 10 million new cases of cancer are diagnosed every year, and the statistical trends indicate that this number would double by 2020.[4] Studies and practice in Eastern medicine have shown that certain mushrooms have extra ordinary effects on people dealing with various types of cancer, and some of them have been used since the 16th or 17th century.[5,6]

Regarding food preserving properties, a number of studies have been recently carried out with success,[7-9] being evident the considerable differences among the chemical composition, nutritional value and bioactive effects of the mushroom species. Therefore, a detailed chemical analysis of each source of bioactive compounds is necessary.

On the other hand increasing evidences that artificial preservatives (*e.g.*, butylated hydroxyanisole- BHA, butylated hydroxytoluene- BHT and tertiary butyl hydroquinone- TBHQ) are carcinogenic substances, pressured food industry to start searching for novel substances to prolong shelf life of products. Accordingly, natural sources appeared as excellent alternatives to replace synthetic antioxidants.[10,11]

Meat and meat products are very susceptible to spoilage due to microbial growth, oxidation and enzymatic autolysis. Traditional methods such as salting, drying, smoking, are still used,[12] but they do not meet standards of novel consumers who demand natural ingredients as preservatives. Bearing in mind that food spoilage is related to growth of microorganisms and free radicals production, natural sources of antioxidant and antimicrobial compounds should be explored. *Laetiporus sulphureus* (Bull.: Fr.) Murr. is a suitable candidate for any survey because it can be easily found and recognized in nature, but it can also be easily cultivated.[13] Therefore, this edible mushroom is considered as a promising object for biotechnology studies.

The objective of the present study was to evaluate the chemical composition of *L. sulphureus* and *in vitro* bioactive properties namely, antioxidant, antitumor and antimicrobial of its methanolic and polysaccharidic extracts. Furthermore, the preservative efficiency of the methanolic extractwas studied using chicken pate as model system and the mould *A. flavus* as contaminant fungus. It should be highlighted that this study was carried out under *in situ* conditions, using actual foods and usual storage conditions, rather than microbiological media (*in vitro* systems), which is a huge advantage in relation to other available studies regarding meat preserving activity through natural matrices.

Materials and Methods

Mushroom species

L. sulphureus was collected from the trees of *Salix alba* in Jojkićev branch of Danube River, near Pančevo, Northern Serbia, during May of 2011 and authenticated by Dr. Jasmina Glamočlija (Institute for Biological Research). A voucher specimen has been deposited at the Fungal Collection Unit of the Mycological Laboratory, Department for Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia, under number Ls-610-2011. Fresh fruiting bodies were randomly divided to smaller samples and immediately dried by lyophilization (LH Leybold, Lyovac GT2, Frenkendorf). When reaching constant mass, specimens were milled to a fine powder, mixed to obtain an homogenate sample, and kept at 4ºC untill further analysis.

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 tocopherols, sugars, organic acids and phenolic compounds, 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). Mueller-Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia).

Dimethylsulfoxide (DMSO), (Merck KGaA, Germany) was used as a solvent. Phosphate buffered saline (PBS) was obtained from Sigma Chemical Co. (St. Louis, USA). Foetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), non-essential amino acids solution (2 mM), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, USA). Acetic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Chemical Co. (Saint Louis, USA). Methanol and all other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

Chemical characterization of *L. sulphureus*

Nutritional value

The samples were analyzed for chemical composition (moisture, proteins, fat, carbohydrates and ash) using the procedures described by the Association of Official Analytical Chemists (AOAC).[14] 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 their difference. Energy was calculated according to the following equation: energy (kcal) = 4 × (g protein + g carbohydrate) + 9 × (g fat).

Sugars composition

Free sugars were determined by a high performance liquid chromatography (HPLC) system consisted of an integrated system with a pump (Knauer, Smartline system1000), degasser system (Smartline manager 5000) and an auto-sampler (AS-2057 Jasco), coupled to a refraction index detector (RI detector KnauerSmartline 2300) as previously described by the authors.[15] 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 g/100 g of dry weight.

Fatty acids composition

Fatty acids were determined after a transesterification procedure as described previously by the authors,[15] 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.

Tocopherols composition

Tocopherols were determined following a procedure previously optimized and described by the authors.[16] 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 μg/100 g of dry weight.

Organic acids composition

Organic acids were determined following a procedure previously optimized and described by the authors.[17] 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 nm 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/100 g of dry weight.

Phenolic compounds composition

Phenolic acids were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA) as previously described by Barros et al.[18] Detection was carried out in a diode array detector (DAD) using 280 nm as the preferred wavelength. The phenolic compounds 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/100 g of dry weight.

Preparation of methanolic and polysaccharidic extracts

Powder of *L. sulphureus* (10 g) was extracted by stirring with 250 mL of methanol at -20 °C for 6h. The extract was sonificated for 15 minutes, centrifuged at 4000 g for 10 minutes, and subsequently filtered through Whatman No.4 paper. The residue was then extracted with two additional portions of methanol (each time l50 mL of solvent). The combined extract was evaporated at 40 °C (rotary evaporator Büchi R-210) to dryness and re-dissolved in 30% ethanol. [18] The yield of methanolic extract was 9.8%.

Polysaccharides were extracted from powder mushroom (1.5 g) by stirring with boiling water (50 mL) for 2h before being subsequently filtered through Whatman No. 4 paper. The residue was then extracted with two further portions of boiling water over a total 6 h extraction. The combined extracts were lyophilized before 95% ethanol (10 mL) was added and polysaccharides were precipitated overnight at 4 ºC. The precipitated polysaccharides were collected after centrifugation at 3.100 g for 40 min followed by filtration, before being lyophilized, resulting in a crude polysaccharidic sample.[19] The yield of the extract was 2.5%.

The extracts were redissolved in *i)* methanol or water (final concentration 20 mg/mL) for antioxidant activity evaluation, and *ii)* water for antitumor (final concentration 8 mg/mL) and antimicrobial (final concentration 1.5 mg/mL) activity evaluation. The final solutions were further diluted to different concentrations to be submitted to distinct bioactivity evaluation *in*

206 *vitro* assays. The results were expressed in *i)* EC_{50} values (sample concentration providing 50%
207 of antioxidant activity or 0.5 of absorbance in the reducing power assay) for antioxidant activity;
208 *ii)* GI_{50} values (sample concentration that inhibited 50% of the net cell growth) for
209 antiproliferative activity in human tumor cell lines and non-tumor liver primary cell culture; and
210 *iii)*MIC (Minimum inhibitory concentration) and MBC/MFC (Minimum bactericidal
211 concentration/Minimum fungicidal concentration) values for antimicrobial activity. Trolox and
212 ellipticine were used as positive controls in antioxidant and antitumor activity evaluation assays,
213 respectively. Streptomycin and ampicillin were used as positive controls in the antibacterial
214 assay, while bifonazole and ketokonazole were used as positive controls in the antifungal
215 susceptibility test.
216
217 Antioxidant activity of *L. sulphureus* methanolic and polysaccharidic extracts
218 DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity
219
220 The reaction mixture on 96 wells plate consisted of a solution by well of the extract solutions
221 with different concentrations (30 µL) and methanol solution (270 µL) containing DPPH radicals
222 (6×10^{-5} mol/L). The mixture was left to stand for 30 min in the dark, and the absorption was
223 measured at 515 nm ELX800 Microplate Reader (Bio-Tek Instruments).[20] The radical
224 scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the
225 equation:% RSA=[(ADPPH-AS)/ADPPH]×100, where AS is the absorbance of the solution
226 containing the sample, and ADPPH is the absorbance of the DPPH solution.
227
228 Reducing power

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 50 °C for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48 wells plate, the same with deionized water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm in the microplate reader mentioned above.[20]

β-carotene bleaching inhibition

A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two milliliters of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40 °C under vacuum and linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into test tubes containing extract solutions 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.[20] β-carotene bleaching inhibition was calculated using the following equation: (Absorbance after 2 h of assay/initial absorbance)×100.

TBARS (thiobarbituric acid reactive substances) inhibition

Porcine (*Susscrofa*) 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 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 500 μL), followed by thiobarbituric acid (TBA 2%, w/v, 380 μL), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000 g for 10 min to remove the precipitated protein, the color intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm.[20] 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.

Antiproliferative activity of *L. sulphureus* methanolic and polysaccharidic extracts against human tumor cell lines and non-tumor liver primary cells.

Five human tumor cell lines were tested: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HCT-15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS (MCF-7,NCI-H460 and HCT-15) and 2 mM glutamine or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (HeLa and $HepG_2$ cells), at 37ºC, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3cells/well for MCF-7, NCI-H460 and HCT-15 or 1.0×10^4cells/well for HeLa and $HepG_2$) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48 h with the diluted fraction/extract

solutions. Following this incubation period, the adherent cells were fixed by adding cold 10% trichloroacetic acid (TCA, 100 µL) and incubated for 60 min at 4 ºC. Plates were then washed with deionized water and dried; sulforhodamine B solution (0.1% in 1% acetic acid, 100 µL) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried, the bound SRB was solubilised with 10 mM Tris (200 µL, pH 7.4) and the absorbance was measured at 540 nm in the microplate reader mentioned above.[21]

A cell culture was also prepared from a freshly harvested porcine liver obtained from a local slaughter house. It was designed 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^2 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 37ºC 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.[22] Cells were treated for 48h with the different diluted sample solutions and the same procedure described for SRB assay was followed.

Antimicrobial activity of *L. sulphureus* methanolic and polysaccharidic extracts

Antibacterial activity assays

The following Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), and Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *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. Briefly, fresh overnight culture of bacteria was adjusted by the spectrophotometer 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 extract solutions were carried out over the wells containing 100 µL of Tryptic Soy Broth (TSB) and afterwards, 10 µL of inoculum was added to all the wells. The microplates were incubated for 24h 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 of the growth of the bacteria in comparison with the positive control was identified as the MIC. The MICs obtained from the susceptibility testing of various bacteria to tested extract 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.[23,24] 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. 5% DMSO was used as negative control.

Antifungal activity assays

For the antifungal bioassays, the following microfungi were used: *Aspergillus fumigatus* (ATCC 1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Trichoderma viride* (IAM 5061), *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.[25]

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

MICs determination was performed by a serial dilution technique using 96-well microtitre plates. The solution extracts were 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. Five % DMSO was used as a negative control.[26]

344

345 Chicken pate

346

347 Chicken pate, produced from the chicken meat by Neoplanta, Novi Sad, Serbia was purchased
348 from a supermarket and kept unopened in the refrigerator at 4°C until further analyses. Upon
349 opening the pate was immediately used in the experiments. The sample was used before the
350 expiry date of the product. On the label is clearly defined the nutritional value per 100 g of the
351 product. Composition of the chicken pate stated on the packaging was: proteins 11 g, fats 21 g,
352 carbohydrates 3 g, energy value per 100 g 253 kcal. The packaging stated no artificial
353 preservatives. Experiments on inoculating Malt Agar (MA) and Muller–Hinton Agar (MHA)
354 plates with pate diluted by 1 in 10 with phosphatebuffered saline (PBS) and kept at 25 °C and 37
355 °C, for 48 h, showed no bacterial or fungal contamination of the product.

356

357 Isolation and identification of chicken pate contaminant micromycete

358

359 Chicken pate was left open at 4 °C for 30 days. After that period, the experiment was repeated
360 with dilutions to check for contaminants. MA plates with micromycete were inoculated with
361 different dilutions of pate in PBS and kept at 25°C in order to investigate possible fungal
362 contaminants. Pure fungal culture was isolated from inoculated MA plates after 48 h and
363 identified by Dr. Milica Ljaljević-Grbić (Faculty of Biology, University of Belgrade) as
364 *Aspergillus flavus*. Specimen was deposited in the Micromycetes Collection Unit of the
365 Mycological Laboratory, Department for Plant Physiology, Institute for Biological Research
366 "Siniša Stanković", Belgrade, Serbia, under number Af-CP-01.

In situ antifungal assay in chicken pate

Prior to the experiment, chicken pate was held refrigerated. Packaging was carefully opened in sterile conditions. Surface of the product was prepared for the inoculation by making wells (3 mm deep and 3 mm wide) with a sterile nail, with three wells per each packaging. Each treatment had two replicates with five packaging per replicate. Methanolic mushroom extract wasdissolved in different concentrations in sterile saline containing 0.2% Tween 80 (v/v). Then 20 µL of the extract was placed into each well. After 1h, 20 µL of a conidial suspension (10^6 CFU/mL) of *A. flavus* was added to each well. Treated chicken pate products were put in sterile plastic boxes. The treated samples were stored at 4°C for 21 days. After storage period, the number of wells that showed contamination was recorded and contamination incidence (%) was calculated as follows:

$$Contamination\ incidence\ (\%) = \frac{Number\ of\ contamined\ wells}{Number\ of\ total\ wells\ per\ replicate}\ x\ 100$$

Inhibition percentage was also calculated, by comparing growth in non-treated control samples with treated samples that possessed growth of the mould. Diametrs of the growth zones in control samples (measured on 0 day, 7th day, 14th day and 21th day) were used as 100% growth, while growth inhibition in treated samples was calculated as follows and the results were presented graphically:

$$Growth\ inhibition\ (\%) = \frac{Diameter\ of\ growth\ in\ treated\ sample}{Diameter\ of\ growth\ in\ control\ sample}\ x\ 100$$

Results and Discussion

Chemical composition

Our literature survey showed that there are avalialble studies concerning *L. sulphureus* polysaccharides,[13] enzymes,[27] and pigments,[28] but not inthe chemical parameters analysed herein.

Results regarding the nutritional value of *L. sulphureus* are presented in **Table 1**. Carbohydrates and proteins are the most abundant compounds (72.64 g/100 g dw and 15.97 g/100 g dw, respectively). Mushrooms are generally considered to be a good source of digestable proteins, and are reported to contain all the essential amino acids needed in the human diet.[29] Ash content is reported to be low (9.03g/100 g dw). *L. sulphureus* is also poor in fat content (2.35 g/100 g dw) and caloric value (375 kcal/100 g dw), which makes this mushroom a good candidate for low-caloric diets. Trehalose was the dominant sugar (4 g/100g dw), nearly followed by mannitol (3.54 g/100g dw) (**Figure 1A**). This is in concordance with previous reports on mushroom sugars composition.[30]

Linoleic acid (C18:2n6c) was the most abundant fatty acid (**Table 1**, 63.27%), followed by oleic acid (C18:1n9c, 14.52%), and palmitic acid (C16:0, 11.68%) (**Figure 1B**). *L. sulphureus* is rich in polyunsaturated fatty acids- PUFA (64.14%), while saturated and monounsaturated fatty acids are present in lower amounts (20.54% and 15.32%, respectively). As already mentioned, mushrooms are known for their low fat content, but also for high proportion of PUFA, some of them essential fatty acids, which makes them healthy foods.[30,31]

Tocopherols were also determined, since these compounds have the important ability to scavenge free radicals, and are considered to protect our organisms against degenerative diseases. For a long time, α-tocopherol was considered to be the most active form of vitamin E and was reported to have the highest biological activity. However, recent studies have shown that the other forms are also active.[16] For the studied mushroom, α-tocopherol was the most abundant isoform (**Table 2**, 109.25 μg/100 g dw), followed by γ-tocopherol (62.07 μg/100 g dw) and δ-tocopherol (18.42 μg/100 g dw); β-tocopherol was not present (**Figure 2A**).

Regarding organic acids composition (**Figure 2B**), it was possible to determine and quantify four compounds (**Table 2**): oxalic (2.66 g/100 g dw), citric (1.24 g/100 g dw), fumaric (0.25 g/100g dw), and quinic (0.16 g/100g dw) acids. Oxalic and fumaric acids are known for their bioactivities namely, antibacterial, antitumor and antifungal activities.[32-34]

Antioxidant molecules, such as cinnamic (175.28 μg/100 g) and *p*-hydroxybenzoic (30.71 μg/100 g) acids (**Figure 2C**), were also identified and quantified after methanolic extraction (**Table 2**).

In vitro antioxidant, antitumor and antimicrobial properties

Antioxidant activity was measured by four different methods, using methanolic and polysaccharidic extracts. These assays measured free radical scavenging activity, reducing power and lipid peroxidation inhibition. In three of the four assays, polysaccharidic extract exhibited higher activity (lower EC_{50} values) (**Table 3**). Nevertheless, for TBARS assay, it was the methanolic extract that showed the highest activity (EC_{50} = 0.78mg/mL). The same behavior was previously reported for other mushroom species.[35] The observed antioxidant activity may be the

consequence of the presence of different antioxidant compounds described in the previous section such as tocopherols (mainly α-tocopherol), organic acids and phenolic compounds. Other authors previously reported high antioxidant activity of *L. sulphureus* ethanolic and polysaccharidic extracts.[36,37] There are other studies reporting natural products as good antioxidant agents.[38,39]

The results obtained for antiproliferative activity against human tumor cell lines and non-tumor liver primary cells are presented in **Table 4**. The polysaccharidic extract was the only one that showed *in vitro* antitumor activity; nonetheless, it did not show activity against HepG2 cell line. The most susceptible cell lines were HCT15 (GI_{50}= 68.96 μg/mL) and HeLa (GI_{50}= 72.26 μg/mL). None of the extracts were toxic for porcine liver cells (GI_{50}> 400 μg/mL). Previous studies based on natural matrices were published recently reporting antitumor activity of natural products.[39,40]

The polysaccharidic extract also showed higher antibacterial activity than methanolic extract, except against *M. flavus* and *S. typhimurium.* The same was observed for antifungal properties, with the exception of *P. ochrochloron* (**Table 5**). Comparing antibacterial and antifungal activities of the tested extracts, it could be noticed that bacteria were more sensitive to both extracts than fungi. Higher sensitivity of bacteria could be attributed to the different cell organization between bacteria and microfungi (prokaryotic vs. eukaryotic cells). The antibacterial effect of *L. sulphureus* ethanolic extract was also previously reported by Turkoglu et al.[36]

In situ food preserving properties

Data regarding *in situ* preserving properties by *L. sulphureus* methanolic extract are presented in **Tables 6**. Contamination incidence (**Table 6**) was recorded and the results indicate no contamination at concentration of 3.56 mg/well or higher. It was obvious that inhibition of *A. flavus* growth in pate is dose dependent, and that higher concentrations of *L. sulphureus* extract completely inhibited mycelial growth (**Table 6**). It was evident that lower storage temperature slow down the mycelial growth rather than room temperature (approximately 21ºC), but refrigerated conditions were used because it is an appropriate way to storage meat pate. First observations of the mycelial growth of *A. flavus* were registered in control group (without methanolic extract) after 7 days of storage. In groups with the incorporated methanolic extract in different concentrations, it was noted that all the tested concentrations successfully inhibited the growth of the food-poisoning mould during the first 14 days of storage, but in the next seven days, only higher concentrations (3.56 mg/well, 4.75 mg/well, 5.94 mg/well) were sufficiently successful in preventing the micromycete growth. Observation of mycelial growth, spore germination and measurement of the growth inhibition zones was discontinued after 21 days of storage. This decision was made due to the fact that, at the mentioned time, the control group was completely infected and overgrown with *A. flavus*. Therefore, it was determined that 21 days of storage were sufficient for *A. flavus* to fully grow and germinate under exposed conditions of storage. The highest doses used completely retarded the growth of *A. flavus* with inhibition percentage of 100%.

Meat products are widely consumed among people and, therefore, the question of their toxicity is attracking high attention. The reason for this is the knowledge that moulds, which are widely present in meat products, can produce secondary metabolites –mycotoxins– that are not subjected to heat treatment and are implemented through the food chain without any chemical

change. Mycotoxins are known for their unfavorable effects: carcinogenesis, mutagenicity, and high thermostability.[41] *A. flavus* is among the most common pathogens of meat products, especially in those which have ripened for a long time, but this number goes as high as 78 species of moulds which have been isolated from various meat products. Nevertheless, it should be highlighted that the presence of mould alone doesn't mean the presence of mycotoxins. According to Mižáková et al.,[41] several conditions have to be satisfied for moulds to produce mycotoxins, and they are: presence of oxygene, temperature between 4 ºC and 40 ºC, pH value between 2.5 and 8, minimum water activity of 0.80 and maximum salt concentration of 14 %. Spores of moulds are everywhere around us, and they enable the moulds to survive extreme conditions and wait prepared for the favorable conditions to fully develop. This is the reason why they are practically impossible to eliminate from the products even when respecting a very high level of hygiene during the production and storage. Prevention of moulds development is highly recommendable at all stages of meat production from slaughtering of animals to packaging of the final product.[41]

Since consumers have greater awareness and higher concern regarding synthetic chemical additives, food products preserved with natural additives are becoming more popular.[42] Beacuse of that, we have chosen a mushroom with proven antimicrobial and antioxidant properties that could brings functional and preserving properties to meat products, affecting their taste in a positive way and making them acceptable to the customer. In any case, when choosing a natural antioxidant that will be used for purposes of prolonging the shelf life of food, the impact on the sensory and quality of the taste should also be taken into account to obtain a product with desired properties.

Regarding preservation of meat pate with natural substances, another study was carried out by Hayrapetyan et al.[43] using pomegranate extract as inhibitor of bacterial growth. This extract turned out to be an excellent food product preservative. Nevertheless, we think that mushrooms are also good candidates due to their exquisite taste.

Conclusion

Mushrooms traditionally could be used because of their medicinal properties with great potential for therapeutic applications in the treatment of some of the aforementioned diseases. In this work we showed that mushrooms also could be a great source of natural bioactive compounds, including antioxidants, such as polyphenols, vitamins, unsaturated fatty acids and sugars, which can be useful for various applications, especially as food additives and in health promotion as ingredients in formulations of functional foods and nutraceuticals.

Acknowledgements

The authors are grateful to Foundation for Science and Technology (FCT, Portugal) and COMPETE/QREN/EU for financial support to this work (research project PTDC/AGR-ALI/110062/2009 and L. Barros researcher contract under "Programa Compromisso com Ciência-2008") and to CIMO (strategic project PEst-OE/AGR/UI0690/2011). The authors also thank to Serbian Ministry of Education and Science for financial support (grant number 173032) and to Mr. Dragoljub Ratkovic for providing us fresh material of mushroom *Laetiporus sulphureus.*

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526 **Conflict of interest**

527 The authors declare no conflict of interest.

528 This article does not contain any studies with human or animal subjects.

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690 **Table 1.** Nutritional value, sugars and fatty acids composition of *L. sulphureus* (mean ±SD).

Nutritional parameter	**Amount**
Fat	2.35± 0.50 g/100 g dw
Proteins	15.97± 0.13 g/100 g dw
Ash	9.03± 0.82 g/100 g dw
Carbohydrates	72.64± 0.88 g/100 g dw
Energy	375.62± 0.54 kcal/100 g dw
Free sugars	**Amount (g/100 g dw)**
Fructose	0.46±0.02
Mannitol	3.54±0.06
Trehalose	4.00±0.12
Total Sugars	8.00±0.17
Fatty acids	**Percentage (%)**
C6:0 Caproic acid	0.08±0.01
C8:0 Caprylic acid	0.10±0.00
C10:0 Capric acid	0.07±0.00
C12:0 Lauric acid	0.12±0.00
C14:0 Myristic acid	0.47±0.01

C14:1 Myristoleic acid	0.00±0.00
C15:0 Pentadecanoicacid	1.41±0.03
C16:0 Palmitic acid	11.68±0.19
C16:1 Palmitoleic acid	0.19±0.00
C17:0 Heptadecanoic acid	1.94±0.04
C18:0 Stearic acid	3.17±0.02
C18:1n9c Oleic acid	14.52±0.02
C18:2n6c Linoleic acid	63.27±0.26
C18:3n3 Linolenic acid	0.17±0.01
C20:0 Arachidic acid	0.19±0.00
C20:1 *cis*-11-Eicosenoic acid	0.51±0.01
C20:2 *cis*-11,14-Eicosadienoic acid	0.12±0.01
C20:3n3+C21:0 *cis*-11,14,17-Eicosatrienoic acid + Heneicosanoic acid	0.17±0.01
C20:5n3 *cis*-5,8,11,14,17-Eicosapentaenoic acid	0.40±0.02
C22:0 Behenic acid	0.43±0.01
C22:1n9 Erucic acid	0.04±0.01
C23:0 Tricosanoic acid	0.15±0.00
C24:0 Lignoceric acid	0.74±0.00
C24:1 Ceramide	0.07±0.00
SFA	20.54±0.27
MUFA	15.32±0.04
PUFA	64.14±0.23

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692 SFA- saturated fatty acids; MUFA- monounsaturated fatty acids; PUFA- polyunsaturated fatty
693 acids, dw = dry weight.
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695 **Table 2.** Tocopherols and organic acids and phenolic compounds composition of *L. sulphureus*

696 (mean ±SD).

Tocopherols (µg/100 g per dry weight)				
α-tocopherol	γ-tocopherol	δ-tocopherol		Total
109.25±3.08	62.07±2.70	18.42±1.31		189.91±0.93
Organic acids (g/100 g per dry weight)				
Oxalic acid	Quinic acid	Citric acid	Fumaric acid	Total
2.66±0.03	0.16±0.01	1.24±0.06	0.25±0.00	4.31±0.01
Phenolic and related compounds (µg/100 g per dry weight)				
p-Hydroxybenzoic acid		Cinnamic acid		Total
30.71±0.20		144.57±1.82		175.28±1.62

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Table 3. *In vitro* antioxidant potential of *L. sulphureus* (mean ±SD).

EC_{50} values (mg/mL)	Methanolic extract	Polysaccharidic extract	Trolox
DPPH scavenging activity	6.80±0.18	2.18±0.10	0.04 ± 0.00
Reducing power	1.93 ± 0.02	0.72±0.03	0.03 ± 0.00
β-carotene bleaching inhibition	3.56±0.08	0.60±0.02	0.003 ± 0.00
TBARS inhibition	0.78 ± 0.07	4.35±0.23	0.004 ± 0.00

The results are presented in EC_{50} values, meaning that lower values correspond to higher reducing power or antioxidant potential.EC_{50}- extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance for the reducing power assay.

Table 4. *In vitro* antiproliferative activity of *L. sulphureus* against human tumor cell lines and non-tumor liver primary cells (mean ±SD).

GI_{50}values (µg/mL)	Methanolic extract	Polysaccharidic extract	Ellipticine
MCF-7 (breast carcinoma)	>400	239.57±2.44	0.91±0.04
NCI-H460 (non-small cell lung cancer)	>400	181.49±12.58	1.42±0.00
HCT15 (colon carcinoma)	>400	68.96±6.00	1.91±0.06
HeLa (cervical carcinoma)	>400	72.26±5.37	1.14±0.21
HepG2 (hepatocellular carcinoma)	>400	>400	3.22±0.67
PLP2 (non-tumor liver primary cells)	>400	>400	2.06±0.03

GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2.

727 **Table 5.** Antimicrobial activity of *L. sulphureus* (mean±SD).

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Bacteria	Methanolic extract MIC (mg/mL) MBC (mg/mL)	Polysaccharidic extract MIC (mg/mL) MBC (mg/mL)	Ampicillin MIC (mg/mL) MBC (mg/mL)	Streptomycin MIC (mg/mL) MBC (mg/mL)
Micrococcus flavus	1.4±0.06 1.8±0.10	3.1±0.06 6.2±0.10	0.3±0.03 0.4±0.00	0.2±0.02 0.3±0.02
Staphylococcus aureus	1.8±0.00 3.6±0.06	0.4±0.00 2.4±0.06	0.3±0.05 0.4±0.02	0.05±0.00 0.1±0.00
Listeria monocytogenes	3.6±0.00 7.2±0.10	3.1±0.00 6.2±0.10	0.4±0.01 0.5±0.03	0.2±0.01 0.3±0.00
Escherichia coli	2.8±0.03 3.6±0.00	1.5±0.03 3.1±0.06	0.3±0.05 0.5±0.05	0.2±0.03 0.3±0.02
Pseudomonas aeruginosa	2.8±0.03 3.6±0.06	1.5±0.03 3.1±0.06	0.8±0.02 1.25±0.00	0.2±0.04 0.3±0.03
Salmonella typhimurium	0.9±0.02 1.8±0.00	0.4±0.00 4.8±0.20	0.3±0.03 0.4±0.00	0.2±0.00 0.3±0.01
Fungi	Methanolic extract MIC (mg/mL) MFC (mg/mL)	Polysaccharidic extract MIC(mg/mL) MFC (mg/mL)	Bifonazole MIC(mg/mL) MFC(mg/mL)	Ketoconazole MIC(mg/mL) MFC(mg/mL)
Aspergillus fumigatus	2.5±0.10 9.0±0.30	2.0±0.30 4.0±0.20	0.15±0.00 0.20±0.02	0.2±0.00 0.5±0.01
Aspergillus versicolor	4.5±0.10 9.0±0.30	1.0±0.00 4.0±0.03	0.10±0.007 0.20±0.008	0.2±0.06 0.5±0.06
Aspergillus ochraceus	2.5±0.00 5.0±0.10	0.5±0.06 4.0±0.06	0.15±0.02 0.20±0.00	1.5±0.00 2.0±0.03
Trichoderma viride	3.75±0.03 5.0±0.00	2.0±0.00 4.0±0.10	0.10±0.007 0.20±0.03	1.0±0.00 1.0±0.03
Penicillium ochrochloron	1.25±0.06 2.5±0.03	2.0±0.03 4.0±0.10	0.20±0.02 0.25±0.01	2.5±0.20 3.5±0.10
Penicillium verrucosum	4.5±0.06 9.0±0.20	4.0±0.00 8.0±0.20	0.15±0.00 0.20±0.02	0.2±0.03 0.3±0.00

729 **Table 6.** Contamination incidence of *A. flavus* in treated and control samples of chicken pate.

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Mould	Concentration (mg/well)	Contamination incidence (%)
Aspergillus flavus	0	100.0
	1.18	66
	2.38	60
	3.56	0.0
	4.75	0.0
	5.94	0.0

Inhibition zones (mm) (mean±SD) of *A. flavus* in chicken pate with the addition of *L. sulphureus* extract stored at +4º C.

Conc.	Control	1.18 mg	2.38 mg	3.56 mg	4.75 mg	5.49 mg
0 day	0.00	0.00	0.00	0.00	0.00	0.00
7^{th} day	5.00	0.00	0.00	0.00	0.00	0.00
14^{th} day	11.00	0.00	0.00	0.00	0.00	0.00
21^{st} day	25.00	7.35	4.50	0.00	0.00	0.00

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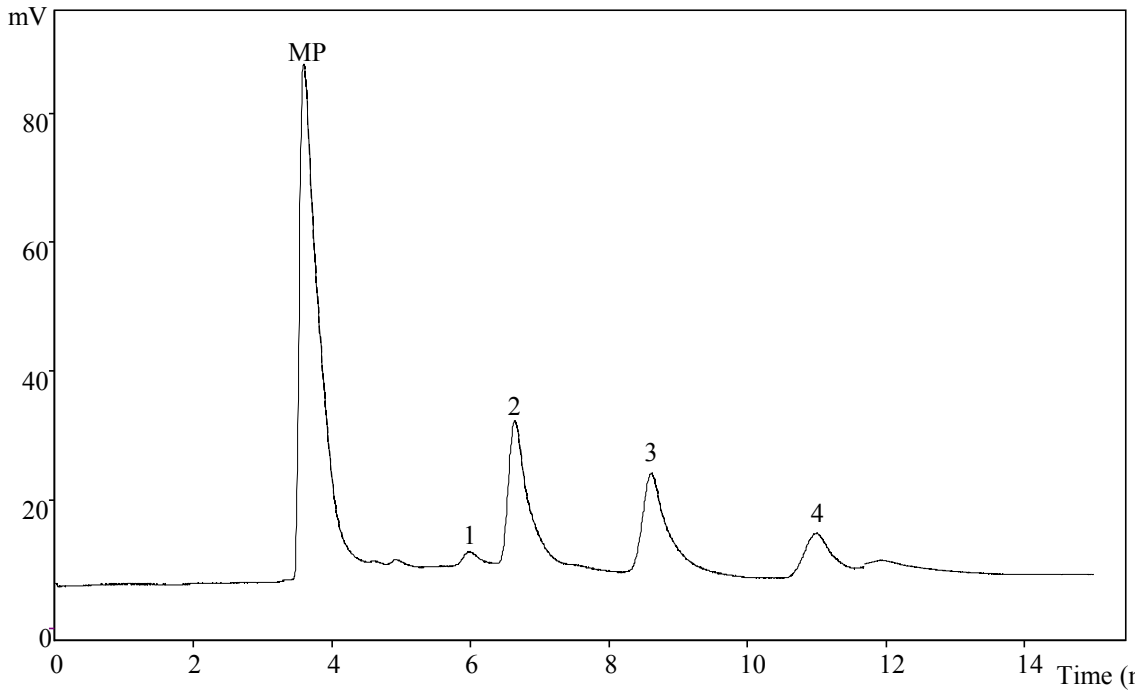
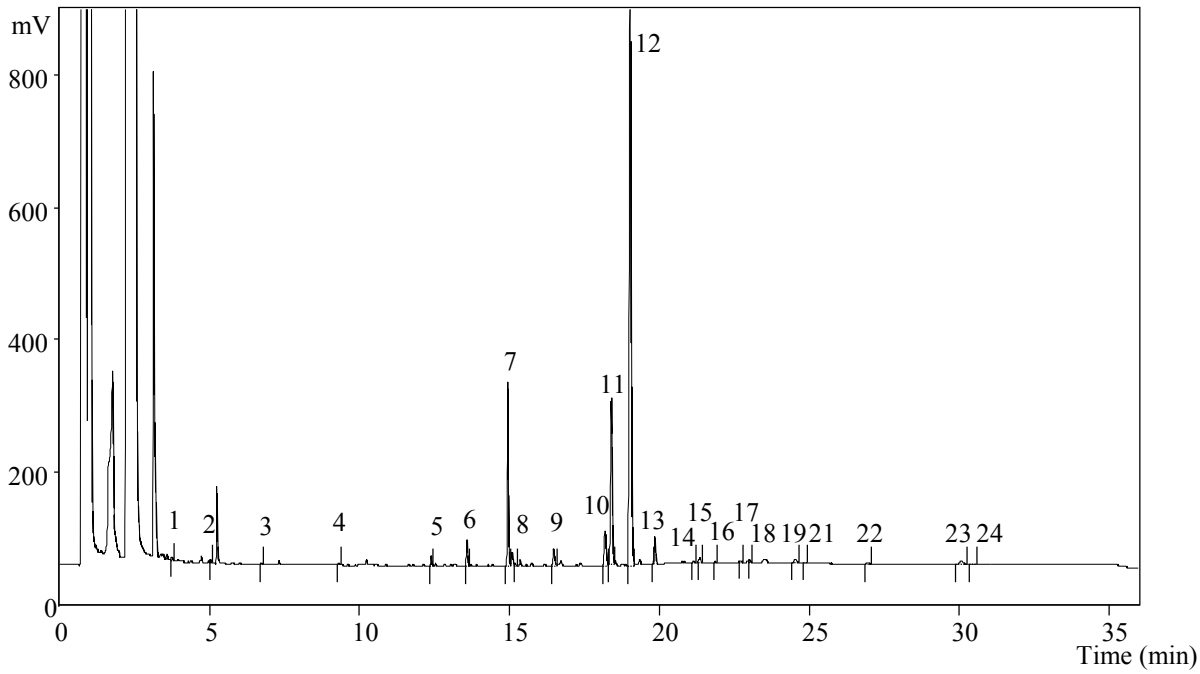
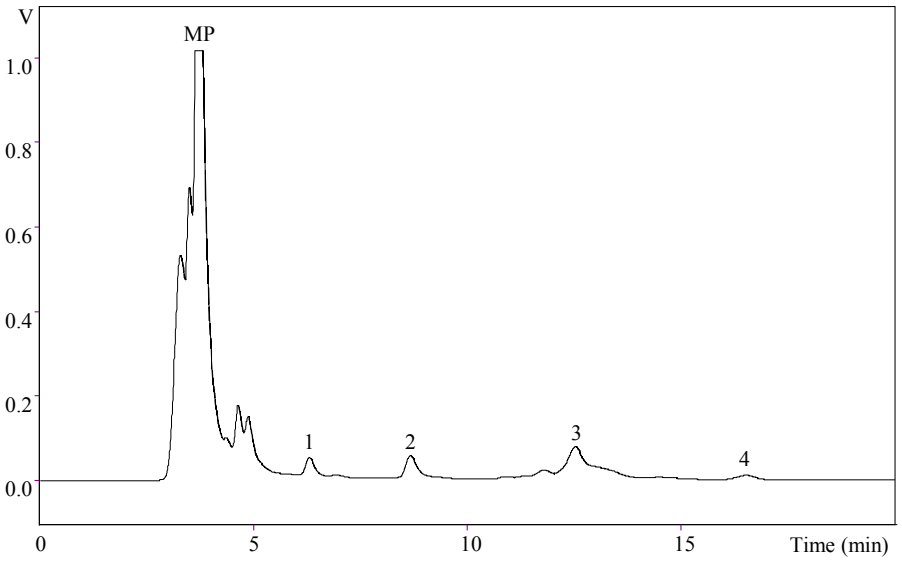


A

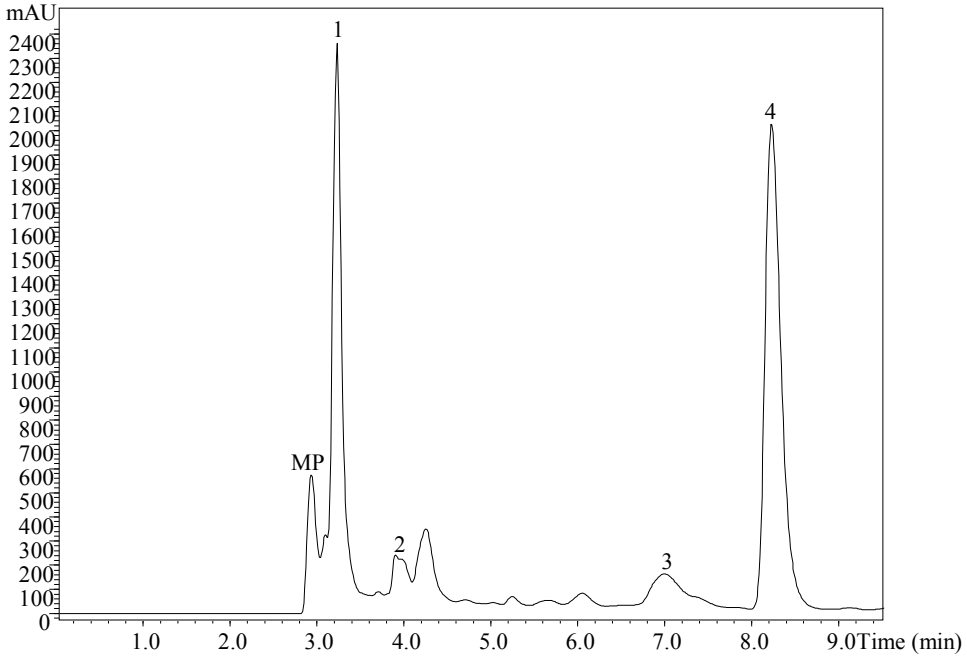


B

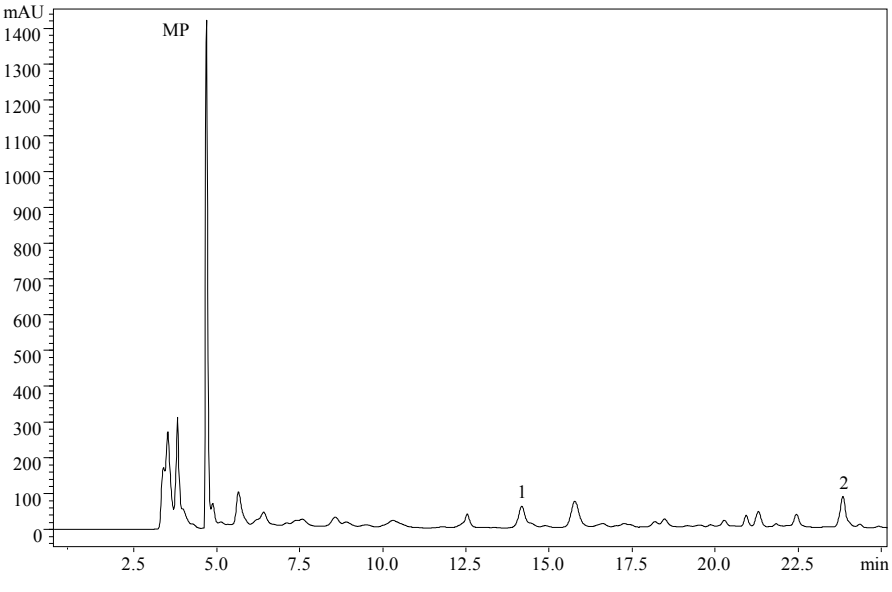
Figure 1. *L. sulphureus* individual profile in (**A**) Sugars: 1- fructose; 2- mannitol; 3- trehalose; 4- raffinose (IS); (**B**) Fatty acids: 1- C6:0; 2- C8:0; 3- C10:0; 4- C12:0; 5- C14:0; 6- C15:0; 7- C16:0; 8- C16:1; 9- C17:0; 10- C18:0; 11- C18:1n9; 12- C18:2n6; 13- C18:3n3; 14- 20:0; 15- C20:1; 16- C20:2; 17- C20:3n3+C21:0; 18- C20:5n3: 19- C22:0; 20 C22:1n9; 21- C23:0; 22- C24:0; 23- C24:1.



742 **A**



743 **B**



C

Figure 2. *L. sulphureus* individual profile in (**A**) Tocopherols: 1- α-tocopherol; 2- γ-tocopherol; 3- δ-tocopherol. Organic acid (**B**): 1- oxalic acid; 2- quinic acid; 3- citric acid; 4- fumaric acid, recorded at 215 nm. (**C**) Phenolic compounds: 1- *p*-hydroxybenzoic acid; 2- cinnamic acid, recorded at 280 nm. MP- mobile phase.