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Chemical and structural characterization of *Pholiota nameko* extracts with biological properties

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ABSTRACT

Edible mushrooms including *Pholiota nameko* are excellent sources of extractable bioactive compounds with much to explore. Enzymatic extractions with Cellulase and Viscozyme were responsible for highest extraction yields (67–77%). No strong antioxidant activity was observed although extracts were able to scavenge $ABTS^{\cdot+}$ and $OH^{\cdot+}$. Potential prebiotic activity was observed in all extracts, some increasing 1.4–2 Log cycles of *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* BB12. 30–50% α-glucosidase inhibition was observed in ultrasound, Flavourzyme and Cellulase extracts. Flavourzyme and Cellulase extracts are richer in macro (Mg, K and P) and micro elements (Zn, Mn and Fe) in comparison to mushroom. Monosaccharides content and profile varied slightly among both extracts with predominance of glucose, galactose and mannose with no uronic acids detection; Flavourzyme extract reported higher free amino acids content. Presence of α and β-glycosidic structures such as glucans and glucan-protein complexes are among the polysaccharides found in both extracts.

1. Introduction

Edible mushrooms are a valuable source of nutrients and of bioactive compounds being increasingly appreciated for their sensory characteristics. Their nutritional value is due to high content of protein, fibre, vitamin and minerals associated to low fat content. Furthermore different bioactive compounds from edible mushrooms have been reported to possess antioxidant, bifidogenic, antitumor, immunomodulatory and antidiabetic activities (Li & Shah, 2016; Li et al., 2015; Quian, Zhang, & Liu, 2015; Zhu et al., 2014a) representing a potential source of functional ingredients.

Extraction and isolation of compounds of interest, able to be ingested or used for food purposes, need to rely upon compatible methods with economically viable yields. Extraction modes tested on mushrooms in the last years include pressurized water extraction (Palanisamy et al., 2014), supercritical fluid extraction (Mazzutti et al., 2012), or extraction with different solvents such as methanol (Moro et al., 2012), ethanol and ethyl acetate (Seephonkai et al., 2012). Water-based extraction is food compatible, non-expensive and environment friendly but, in general, has a low selectivity with low extraction efficiency (Heo, Jeon, Lee, Kim, & Lee, 2003). Considering these limitations, the main objective of this study was to obtain water-based extracts using alternative approaches such as enzyme-assisted extraction (EAE) and ultrasound-assisted extraction (UAE) on cultivated *Pholiota nameko* and subsequently perform their structural, chemical and biological characterisation. Enzymatic assisted extraction is a relatively recent and environment friendly strategy, which is yet scarcely used for the extraction of bioactive compounds from mushrooms; the enzymes hydrolyse cell wall components increasing cell wall permeability resulting in higher extraction yields of solid components (Puri, Sharma, & Barrow, 2012; Zhu et al., 2014b). Ultrasound-assisted extraction is based on sound waves migration which generates cavitation that promote the release of soluble compounds by disruption of cells and their walls enhancing the mass transfer to the extraction solvent (Cravotto et al., 2008) being relatively low-cost. Ultrasound-assisted extraction and EAE have been reported as alternative approaches with great potential to extract bioactive substances from mushrooms (Cheung, Siu, Liu, & Wu, 2012; Tian et al., 2012;

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Yin, You, & Jiang, 2011; Zhu et al., 2014b) but not from *Pholiota nameko*, a wood-rotting fungus widely cultivated in China and Japan but much less in Europe and in USA. Its biological properties have been studied especially in terms of anti-inflammatory, antioxidant, antitumor and immunomodulatory activities (Li, Lu, Zhang, Lu, & Liu, 2008; Li et al., 2015; Quian et al., 2015; Zhang et al., 2015). To our knowledge, this is the first study applying water-based extraction through EAE or UAE on cultivated *Ph. nameko* evaluating chemical, structural and biological properties. The chemical characterization of the extracts was based on analysis of proximate and elemental composition as well as free amino acids and monosaccharides whereas the structural analysis was based on FTIR-ATR and ^{1}H NMR analysis. Biological properties such as antioxidant, prebiotic and antidiabetic activities of the extracts were analysed to assess their potential added value to be used in food and/or nutraceutical applications.

2. Materials and methods

2.1. Specimens and cultivation conditions

Dried specimens of *Pholiota nameko* were supplied by Bioinvitro, Biotecnologia, Lda. (Gandra, Portugal). Mushrooms were cultivated through a standard procedure in filter bags with sterilized organic substrate (76% sawdust beech, 5% fibres (wood, straw), 9% crushed grain corn, 7% wheat bran, 3% crushed oil seed cake with 65% water content) along 30–40 days of incubation at 20–22 °C, followed by a fructification period of 15–21 days at 16–20 °C (information provided by supplier Bioinvitro Lda). After fructification and growth, entire clean mushrooms were dried in a ventilated drier over 24 h between 40 and 60 °C and subsequently milled to less than 1.0 mm using a grinder (Princess Household Appliances, The Netherlands).

2.2. Ultrasound, hot water and enzymatic-assisted extraction

Different water-based extracts of *Ph. nameko* were prepared in triplicate: i) Hot water extraction (HWE) was performed as a control and comparative water based extraction approach; 1 g of dried mushroom was dispersed in 50 mL of deionised water and incubated in an agitated water bath at 50 °C for 24 h. The aqueous solution was then centrifuged at 5000 g for 10 min at 4 °C (centrifuge Medifriger BL-S, JP Selecta, Spain) and the supernatant was filtered through a glass filter funnel (porosity 1) and the resulting extract was frozen at −80 °C until lyophilisation; ii) UAE extracts were prepared as for HWE and after 24 h at 50 °C submersed in a water bath ultrasonicator (Ultrasonik 57H Ney, 400 W, 50/60 Hz) for 60 min (sonicate for 10 min and pause for 2 min) at 50 °C. The resultant aqueous solution was then centrifuged, filtered and frozen according to HWE procedures; iii) For the enzymatic extracts (EA), the same amount of mushroom (1 g) was dispersed in 50 mL of deionised water and incubated in an agitated water bath for 10 min. After adjusting pH to specific enzyme optimum conditions (Alcalase: pH = 8–50 °C; Flavourzyme: pH = 7.0–50 °C; Cellulase: pH = 4.5–50 °C; Viscozyme® L: pH = 4.5–50 °C; All enzymes were obtained from Sigma-Aldrich), 100 mg of enzyme was added and incubated for enzymatic hydrolysis for 24 h at 50 °C. The enzymatic reaction was stopped by heating the sample at 90–100 °C for 10 min followed by immediate cooling in an ice bath. The pH of EA was adjusted to pH 7.0 with 1 M HCl and/or NaOH and then centrifuged, filtered and frozen according to HWE procedures. The frozen extracts were lyophilized, weighed and stored in desiccators in the dark, at room temperature until further study. The extraction yield was based on the ratio between the amounts of lyophilized extract to the amount of extracted dried mushroom.

2.3. Proximate composition of all extracts

All water-based extracts were analysed in triplicate for nitrogen content, total sugar content as well as for total phenolics, according to methods described in Rodrigues et al. (2015a).

2.4. Evaluation of biological properties of all extracts

2.4.1. Antioxidant activity

Total antioxidant capacity of extracts, the concentration of inhibitor required to reduce the activity of the enzyme by 50% (IC_{50}) and the hydroxyl radical ($OH^{\cdot}$) scavenging activity were measured according to the methods described in Rodrigues et al. (2015b).

2.4.2. Prebiotic activity

Potential prebiotic activity of mushroom extracts was evaluated by measuring their impact on the growth of two different probiotic strains namely, *Lactobacillus acidophilus* La-5® and *Bifidobacterium animalis* BB12® (CHR-Hansen, Denmark). Prebiotic activity was assessed by enumeration of viable cell numbers of probiotic strains in MRS broth without glucose but supplemented with each of the extracts (6%) throughout 48 h at 37 °C according to procedures described in Rodrigues et al. (2015b). Strains growth in MRS broth with 6% of glucose or 6% of fructooligosaccharides (FOS) (positive controls) as well as without glucose (negative control) was included.

2.4.3. Antidiabetic activity

The α-glucosidase inhibitory activity was determined in 96-well plates according to the method described in Rodrigues et al. (2015b).

2.5. Chemical characterization of selected extracts of Ph. nameko

For the elemental composition, the measurement of the inorganic elements Mo, B, Zn, P, Cd, Co, Ni, Mn, Fe, Mg, Ca, Cu, Na, Al and K in lyophilized extracts was performed in two steps: microwave-assisted digestion followed by quantification of the 15 elements using an inductively coupled plasma (ICP) optical emission spectrometer (OES) with radial plasma configuration according to procedures described in Rodrigues et al. (2015a). Three replicates were performed for each sample as well as blanks. The organic elements C, H, S and N in lyophilized extracts were quantified using a Truspec 630-200-200 Elemental Analyser (Mönchengladbach, Germany). Triplicate samples of up to 3 mg for each extract were placed under combustion at 1075 °C. Carbon, H and S were detected by infrared absorption whereas N was detected by thermal conductivity.

Monosaccharides, uronic acids and amino-monosaccharides composition was analysed by high performance liquid chromatography (HPLC) after acid hydrolysis. For each lyophilized extract, 2.5 mg of sample was hydrolysed with 2 mL of 2 M trifluoroacetic acid at 110 °C for 4 h. The hydrolysate was then dried by vacuum evaporation at 50 °C and re-dissolved in 2 mL deionised water. The hydrolysate solution (450 μL) was mixed with 450 μL of 1-phenyl-3-methyl-5-pyrazolone solution (0.5 M in methanol) and 450 μL of NaOH solution (0.3 M) and then reacted at 70 °C for 30 min. The reaction was stopped by neutralizing with 450 μL of 0.3 M HCl, and the product was then partitioned with chloroform three times. The aqueous layer was collected and filtered through a 0.45 μM membrane and was applied to HPLC. The HPLC was performed in an Agilent 1100 (Waldbronn, Germany) and a ZORBAX ECLIPSE XDB-C18 column (4.6 × 150 mm, 5 micro) at 25 °C with potassium phosphate buffer saline (0.05 M, pH 6.9) with 15% (solvent A) and 40% acetonitrile (solvent B) as mobile phases and detected by UV detector at 250 nm. All analysis were made in quintuplicate and quantified using a calibration curve built with monosaccharides standards (Sigma Aldrich, St. Louis MO, USA)