Food Chemistry 216 (2017) 176–185

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

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

Dina Rodrigues [a,*], Ana C. Freitas [a,b], Sérgio Sousa [c], Manuela Amorim [c], Marta W. Vasconcelos [c], João P. da Costa [a], Artur M.S. Silva [d], Teresa A.P. Rocha-Santos [a], Armando C. Duarte [a], Ana M.P. Gomes [c]

[a] CESAM – Centre for Environmental and Marine Studies & Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[b] ISEIT/Viseu, Instituto Piaget, Estrada do Alto do Gaio, Galifonge, 3515-776 Lordosa, Viseu, Portugal
[c] Universidade Católica Portuguesa, CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Arquitecto Lobão Vital, Apartado 2511, 4202-401 Porto, Portugal
[d] QOPNA – Organic Chemistry, Natural Products and Food Stuffs Research Unit & Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

ARTICLE INFO

Article history:
Received 18 March 2016
Received in revised form 10 August 2016
Accepted 10 August 2016
Available online 11 August 2016

Keywords:
Pholiota nameko
Enzymatic- and ultrasound-assisted extraction
Chemical characterization
Structural characterization
Prebiotic activity
Antidiabetic activity

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;

* Corresponding author at: CESAM – Centre for Environmental and Marine Studies & Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal.
E-mail address: dinarodrigues@ua.pt (D. Rodrigues).

http://dx.doi.org/10.1016/j.foodchem.2016.08.030
0308-8146/

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)

and expressed as mg/g $_{\text{lyophilized extract}}$. D- and L-Glucose, D- and L-galactose, DL-arabinose, L-fucose, D- and L-mannose, D-xylose, L-rhamnose, D-glucuronic acid, D-galacturonic acid and D-glucosamine-6-phosphate were used as the standards. Recovery ranged between 93 and 99% with a LOD of 0.095 mg/g.

Free amino acids content of each extract was performed by pre-column derivatization with orthophthalaldehyde (OPA) methodology. Isoindole-type fluorescent derivatives were formed in an alkaline solution (borate buffer pH 10.4) from OPA, 2-sulfanylethanol and the primary amine group of the amino acid. The derivatives were separated by HPLC (Beckman coulter, California USA) coupled to a fluorescence detector (Waters, Milford. MA, USA) according to the procedure of Proestos, Loukatos, and Komaitis (2008). 100 μL of each sample, at concentration of 10 mg/mL was derivatised according to the OPA method and injection volume of derivatives was of 20 μL. All analysis were made in triplicate and quantified using a calibration curve built with amino acids pure standards (Sigma Aldrich, St. Louis MO, USA) and expressed as g/100 g of protein content. Recovery ranged between 92 and 99% with a LOD of 0.02 g/100 g of protein content.

2.6. Structural characterization of selected extracts of Ph. nameko

Structural analysis of freeze-fried extracts were analysed by Fourier Transform Infrared Spectroscopy with attenuated total reflectance (FTIR-ATR) according to Rodrigues et al. (2015a). In addition ^{1}H NMR Analysis was performed in each lyophilized extract; 20 mg of extract was suspended in 700 μL of D_2O and subsequently agitated in a vortex for 10 min. 650 μL of the dissolved lyophilized extract was placed in 5 mm NMR tubes (Aldrich528PP, 5 mm). All spectra were acquired on a Bruker Avance 300 spectrometer (Karlsruhe, Germany) with an operating frequency of 300.13 MHz. The acquisition of spectra was performed with a spinning rate of 20 Hz, a contact time of 4.75 s and with the pulse program ZG30. The recycle delay was 1 s and the length of the proton 90 pulses was of 9.00 μs. About 56 scans were collected for each spectrum. A 0.3 Hz line broadening weighting function and baseline correction were applied. The identification of functional groups in the NMR spectra was based on their chemical shift (δ_H) relative to that of the water (4.7 ppm).

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was carried out for each measured parameter, except for prebiotic activity, with SigmaStat™ (Systat Software, Chicago, IL, USA) to assess if extraction method was a significant source of variation ($P = 0.05$). The Holm-Sidak method was used for pair-wise comparisons ($P = 0.05$). In terms of prebiotic activity, for each probiotic strain, a two-way analysis of variance (ANOVA) was carried out with SigmaStat™, to assess whether the extract and incubation time were significant sources of variation ($P = 0.05$). Since significant differences were observed for each factor and interactions, one-way ANOVAs were carried out to observe if the source of carbon (glucose, FOS or extract) was statistically significant. One-way ANOVAs were also performed to evaluate statistical significance of the viable cell numbers after 24 and 48 h, in comparison to values at 0 h.

3. Results and discussion

3.1. Impact of different extraction methods on Ph. nameko and its biological properties

3.1.1. Extraction yield and proximate composition of the extracts

Extraction yields between 54 and 63% were obtained by EAE with proteases, HWE and UAE (Table 1). The lowest yield was obtained with Alcalase whereas the differences between HWE, UAE and EAE with Flavourzyme were not significantly different ($p > 0.05$). Cellulase and Viscozyme assisted extractions were responsible for the statistically significant ($p < 0.05$) highest yields (67–77%). Being fungal cell walls constituted mostly by polysaccharides, comprising about 80% of their dry weight (Ruiz-Herrera, 1991), it is expected that carbohydrases hydrolyse cell wall components thereby increasing their permeability and resulting in higher extraction yields as stated previously by Puri et al. (2012). According to our best of knowledge only a few studies are available with EAE on mushrooms but none applied to *Ph. nameko*. Indeed, Yin et al. (2011) carried out an optimization of polysaccharides extraction from *Tricholoma matsutake* using EAE with a mixture of dual enzyme activities – proteases and carbohydrases (Papain, Pectinase and Cellulase, ratio 1:1:1) testing several parameters but no comparisons were made with other extraction modes. Zhu et al. (2014b) also carried out an optimization of polysaccharides extraction from *Hericium erinaceus* using EAE with a mixture of enzymes (Cellulase, Pectinases and Trypsinase, ratio 2:2:1); the optimal extraction conditions reported resulted in a higher yield (increase of 67.7%) in comparison to those obtained by HWE. In our study, lower extraction yields were achieved using a single enzyme activity. However, they are interesting from the standpoint of the possibility of using different enzymes combination to improve extraction yield.

Ultrasound assisted extraction on *Ph. nameko* did not increase the yield extraction in comparison to HWE. Variable tendencies have been reported for other mushroom polysaccharides yield extraction by UAE which is dependent on mushroom species but also on technological parameters such as ultrasonic power, temperature, time and ratio or water volume to dry biomass (Cheung et al., 2012; Tian et al., 2012). Higher polysaccharides yields were obtained with *Lentinus edodes* and *Agaricus bisporus* but similar or lower extraction yields were reported for *Grifola frondosa* and *Coriolus versicolor* (Cheung et al., 2012; Tian et al., 2012). Physical properties of different mushroom species could be in part responsible for different effects of UAE on the efficiency of polysaccharides extraction.

In general, and as expected, EA obtained with proteases action presented higher nitrogen content (Table 1); Alcalase and

Table 1
Extraction yields and contents of nitrogen, total sugars, and total polyphenols in the different *Ph. nameko* extracts.

Extraction method	Extraction yields ($g_{\text{lyoph extract}}/100g_{\text{dry mushroom}}$)	Nitrogen ($mg/g_{\text{lyoph extract}}$)	Total Sugars ($mg_{\text{glucose equiv}}/g_{\text{lyoph extract}}$)	Total phenolics ($\mu g_{\text{cathecol equiv}}/g_{\text{lyoph extract}}$)
HWE	62 ± 1^{b}	43.7 ± 0.3^{b}	527 ± 32^{b}	153 ± 1^{ab}
UAE	62.86 ± 0.07^{b}	44.0 ± 0.4^{b}	628 ± 33^{c}	144 ± 3^{a}
EAE_Cell	76.8 ± 0.6^{d}	39.5 ± 0.4^{a}	574 ± 20^{bc}	153 ± 13^{a}
EAE_Visc	67.1 ± 0.4^{c}	40.3 ± 0.5^{a}	532 ± 12^{b}	164 ± 9^{ab}
EAE_Alc	54.5 ± 0.7^{a}	51.8 ± 0.5^{d}	376 ± 36^{a}	158 ± 8^{ab}
EAE_Flav	61 ± 2^{b}	46.6 ± 0.3^{c}	526 ± 25^{b}	182 ± 9^{b}

For each parameter, different superscript lowercase letters indicate significant differences ($p < 0.05$) between methods of extraction.

Flavourzyme were responsible for the highest content (47–52 mg/g $_{lyophilized\ extract}$). These values correspond to 73–74% of nitrogen content present in the dry mushroom which was 38.6 mg N/g $_{dry\ mushroom}$ (Rodrigues et al., 2015a). Indeed, proteases were the most effective enzymes for protein recovery and accessibility highlighting the Alcalase endopeptidase activity which resulted in a slightly higher recovery rate.

In terms of total sugar content, UAE extracts presented the highest content with 628 mg $_{glucose\ equiv}$/g $_{lyophilized\ extract}$, followed by EA extracts obtained from carbohydrases action responsible for values of 574 and 531 mg $_{glucose\ equiv}$/g $_{lyophilized\ extract}$ in Cellulase and Viscozyme extracts, respectively. The highest sugar content in UAE extract may result from the mechanical action of ultrasound waves promoting a higher breakdown and release of mushroom cell wall polysaccharides. It must be emphasized that Cellulase was able to extract 74% of total sugars present in the dry mushroom which was 596 mg/g $_{dry\ mushroom}$ (Rodrigues et al., 2015a) whereas 55, 66 and 60% of total sugars were extracted by HWE, UAE and Viscozyme, respectively.

Total phenolics varied slightly with highest values observed in Viscozyme, Alcalase and Flavourzyme extracts; values ranged between 158 and 182 μg$_{cathecol\ equivalent}$/g $_{lyophilized\ extract}$ (Table 1), corresponding to extraction efficiency of only 13–16% of total phenolics present in dry mushroom (675 μg$_{cathecol\ equivalent}$/g$_{lyophilized\ extract}$; Rodrigues et al., 2015a).

3.1.2. Biological properties of the extracts

All extracts were able to scavenge $ABTS^{\cdot+}$ radical (Fig. 1a); at the concentration of 2 mg $_{lyophilized\ extract}$/mL 35–44% scavenging activity was observed for all extracts (Table 1). Enzymatic extracts obtained with proteases registered the highest total antioxidant capacity with 41–44% scavenging activity. Higher concentrated extracts were tested and IC_{50} values of 2.5–2.6 mg $_{lyophilized\ extract}$/mL were obtained for EAE with the proteases, whereas 3.1–3.4 mg $_{lyophilized\ extract}$/mL were needed for the EAE with carbohydrases, HWE and UAE extracts (data not shown). Potential antioxidant activity in water and hot water extracts from *Ph. nameko* were reported by Ji, Zhang, Zhang, Li, and Yang (2012).

In general, a good scavenging capacity for $HO^{\cdot}$ reactive oxygen species was obtained for all 2 mg $_{lyophilized\ extract}$/mL extracts (Fig. 1b). More than 55% scavenging activity was observed with HWE, UAE and Viscozyme extracts. The Cellulase extract had the lowest statistically significant scavenging activity for both the $ABTS^{\cdot+}$ and $OH^{\cdot}$ radicals ($p < 0.05$); an extract characterized by the highest extraction yield, rich in total sugars but with lower values of nitrogen and total phenolics (Table 1). Potential antioxidant effects of polysaccharide extract from *Ph. nameko* have been however reported by Zhang et al. (2015). Although significant correlations are not possible, it seems that the statistically significant difference between the extracts ($p < 0.05$), could be, at least in part, due to protein compounds present in the extracts which may eventually indicate that some protein degradation products may be related to the antioxidant properties. Recently, a ~43 kDa antioxidant protein was isolated from *Ph. nameko* exhibiting potential antioxidant activity (Quian et al., 2015).

Mushrooms are a potential source of prebiotics because they are rich in non-digestible dietary fibres such as glucans, chitin and heteropolysaccharides (Bhakta & Kumar, 2013). Nonetheless, to our best of knowledge, no information is available regarding the prebiotic potential of the *Ph. nameko* or of its extracts. In general, a prebiotic potential of the extracts upon growth of both probiotic strains was observed, when used as the single carbon source (Table 2). Moreover, all extracts were able to increase growth similarly to that promoted by traditional carbon source glucose or prebiotic FOS promoting longer survival stability, except Alcalase extract for *B. animalis* BB12. Significantly higher viable numbers

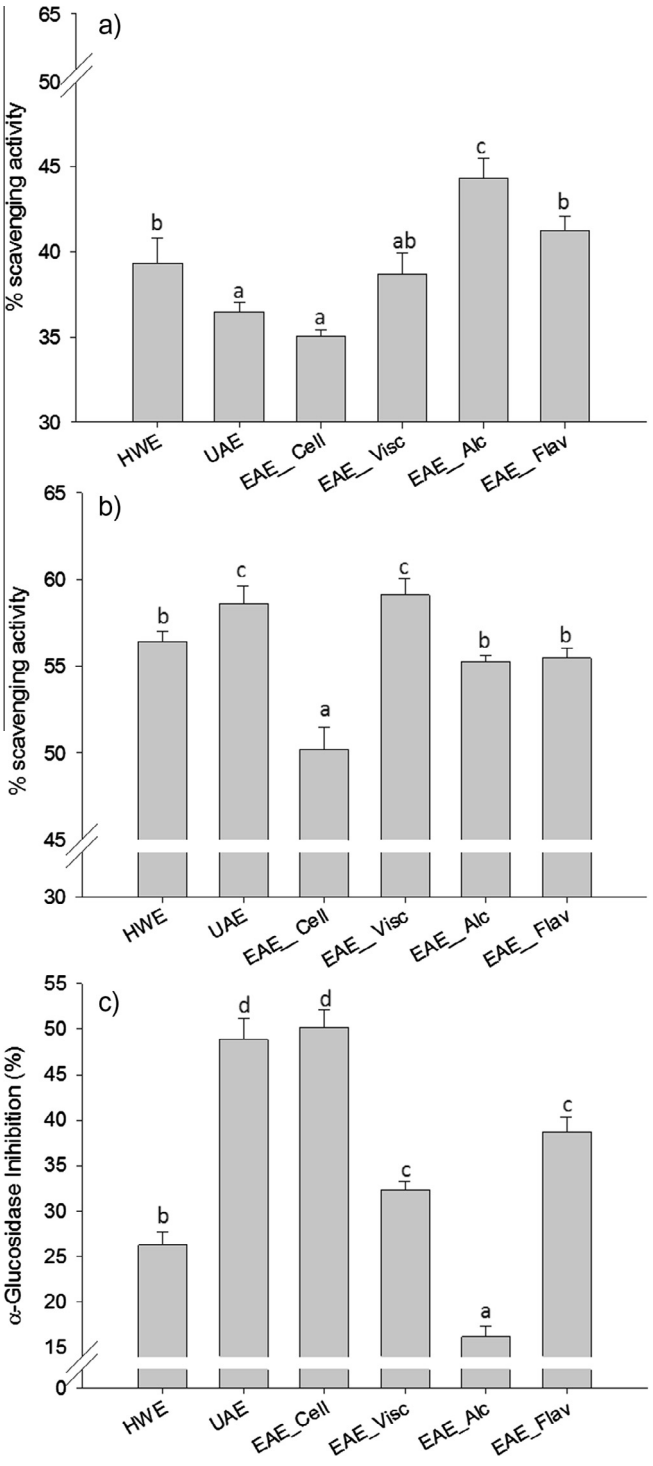

Fig. 1. Total antioxidant capacity (a), hydroxyl radical scavenging activity (b) and α-glucosidase inhibitory activity, expressed as % of inhibition (c) of *Pholiota nameko* extracts obtained by hot water extraction (HWE), ultrasound-assisted extraction (UAE) and enzymatic-assisted extraction (EAE) with Cellulase (Cell), Viscozyme® L (Visc), Alcalase (Alc) and Flavourzyme (Flav) at 50 °C. Different lowercase letters indicate significant differences ($p < 0.05$) between extracts.

($p < 0.05$) of *L. acidophilus* La-5 were observed for all the extracts after 24 and 48 h of incubation in comparison to growth in glucose or in FOS; associated higher growth rates were also observed ($p < 0.05$). An increase of 2 Log cycles was observed with HWE, UAE as well as with Viscozyme and Flavourzyme extracts (Table 2) resulting in increased ratios of 1.3 after 48 h.

Table 2
Mean and standard deviation of cell counts[1] [Log (cfu/mL)] of *L. acidophilus* La-5 and *B. animalis* BB12.

	Time (hrs)	No Sugar Log (cfu/mL)	No Sugar R^2	Glucose Log (cfu/mL)	Glucose R^2	FOS Log (cfu/mL)	FOS R^2	Time (hrs)	HWE Log (cfu/mL)	HWE R^2	UAE Log (cfu/mL)	UAE R^2	EAE_Visc Log (cfu/mL)	EAE_Visc R^2	EAE_Cell Log (cfu/mL)	EAE_Cell R^2	EAE_Alc Log (cfu/mL)	EAE_Alc R^2	EAE_Flav Log (cfu/mL)	EAE_Flav R^2
L. acidophilus La-5	0	7.00 ± 0.08	1.00	7.00 ± 0.08	1.00	7.00 ± 0.08	1.00	0	7.00 ± 0.08	1.00	7.00 ± 0.08	1.00	7.00 ± 0.08	1.00	7.00 ± 0.08	1.00	7.00 ± 0.08	1.00	7.00 ± 0.08	1.00
	4	7.41 ± 0.04	1.06	7.57 ± 0.06	1.08	7.50 ± 0.05	1.07	4	7.6 ± 0.2	1.09	7.72 ± 0.04	1.10	7.6 ± 0.1	1.08	7.62 ± 0.01	1.09	7.48 ± 0.03	1.07	7.55 ± 0.02	1.08
	8	7.92 ± 0.01	1.13	8.11 ± 0.02	1.16	8.0 ± 0.1	1.15	8	8.8 ± 0.1	1.25	8.67 ± 0.04	1.24	8.74 ± 0.09	1.25	8.44 ± 0.08	1.21	8.36 ± 0.02	1.19	8.48 ± 0.03	1.21
	12	5.81 ± 0.01	0.83	5.8 ± 0.4	0.83	7.56 ± 0.01	1.08	12	8.9 ± 0.3	1.27	9.01 ± 0.06	1.29	9.04 ± 0.01	1.29	8.97 ± 0.08	1.28	9.04 ± 0.05	1.29	8.95 ± 0.03	1.28
	24	<3.7[a]	–	3.94 ± 0.07[c]	0.56	5.9 ± 0.2[b]	0.84	24	**9.26 ± 0.01**$_*^{d}$	**1.32**	**9.13 ± 0.01**$_*^{d}$	**1.30**	**9.22 ± 0.01**$_*^{d}$	**1.32**	**9.35 ± 0.04**$_*^{d}$	**1.34**	**9.21 ± 0.04**$_*^{d}$	**1.32**	**9.24 ± 0.07**$_*^{d}$	**1.32**
	48	<3.7[a]	–	4.8 ± 0.2[b]	0.69	6.3 ± 0.8[c]	0.90	48	**9.0 ± 0.2**$_*^{e}$	**1.29**	**8.95 ± 0.04**$_*^{e}$	**1.28**	**9.05 ± 0.04**$_*^{e}$	**1.29**	**8.78 ± 0.01**$_*^{cd}$	**1.29**	**8.61 ± 0.06**$_*^{d}$	**1.23**	**9.06 ± 0.05**$_*^{e}$	**1.29**
B. animalis BB12	0	7.59 ± 0.07	1,00	7.59 ± 0.07	1,00	7.59 ± 0.07	1.00	0	7.59 ± 0.07	1,00	7.59 ± 0.07	1,00	7.59 ± 0.07	1,00	7.59 ± 0.07	1,00	7.59 ± 0.07	1,00	7.59 ± 0.07	1,00
	4	7.3 ± 0.1	0.96	7.8 ± 0.2	1.03	7.58 ± 0.03	1.00	4	7.6 ± 0.1	1.00	7.50 ± 0.03	0.99	7.62 ± 0.02	1.00	7.71 ± 0.01	1.02	7.38 ± 0.04	0.97	7.61 ± 0.01	1.00
	8	7.20 ± 0.05	0.95	8.0 ± 0.3	1.05	8.09 ± 0.07	1.07	8	8.5 ± 0.2	1.12	8.3 ± 0.1	1.09	9.0 ± 0.2	1.18	8.5 ± 0.1	1.12	7.41 ± 0.03	0.98	7.8 ± 0.2	1.03
	12	6.83 ± 0.01	0.90	7.8 ± 0.1	1.03	8.6 ± 0.1	1.13	12	9.08 ± 0.06	1.20	8.95 ± 0.03	1.18	9.3 ± 0.1	1.23	8.79 ± 0.01	1.16	7.68 ± 0.03	1.01	7.9 ± 0.1	1.04
	24	4.5 ± 0.5[a]	0.60	4.9 ± 0.1[a]	0.65	7.8 ± 0.2[bc]	1.03	24	**9.10 ± 0.04**$_*^{cd}$	**1.20**	**9.27 ± 0.02**$_*^{cd}$	**1.22**	**9.36 ± 0.01**$_*^{d}$	**1.23**	**9.0 ± 0.2**$_*^{cd}$	**1.19**	7.4 ± 0.2$_*^{b}$	0.98	**8.46 ± 0.01**$_*^{c}$	**1.11**
	48	<3.7[a]	0.36	5.0 ± 0.8[b]	0.66	5.49 ± 0.03[b]	0.72	48	**9.17 ± 0.05**$_*^{c}$	**1.21**	**9.30 ± 0.01**$_*^{c}$	**1.23**	**9.37 ± 0.01**$_*^{c}$	**1.23**	**9.0 ± 0.1**$_*^{c}$	**1.19**	5.70 ± 0.01$_*^{b}$	**0.75**	**8.44 ± 0.03**$_*^{c}$	**1.11**

[1] Cell counts throughout 48 h of incubation at 37 °C in the presence or absence of glucose, with FOS or with mushroom extract.
[2] Ratio = (Log N_i)/(Log N_0); N = mean (cfu/mL) at time i = 4, 8, 12, 24 or 48 h; N_0 = mean (cfu/mL) at time 0 h; Bold typing for values higher than those obtained with prebiotic FOS after 24 and 48 h and * for significant differences ($p < 0.05$) in comparison to values at 0 h; Different superscript lowercase letters in the same row (24 or 48 h) for each probiotic strain indicate significant differences ($p < 0.05$) for the viable cells between extracts or controls.

Evidence of bifidogenic effect was also observed for *B. animalis* BB12. Viable cell numbers at 24 and 48 h of incubation in all extracts, except in Alcalase, were significantly higher than those obtained with prebiotic FOS and glucose ($p < 0.05$). Similarly to *L. acidophilus* La-5, significant increased growth rates ($p < 0.05$) were also observed. Increases of 1.4 to 1.8 Log cycles were observed with HWE, UAE as well as with Viscozyme and Cellulase extracts (Table 2) resulting in increased ratios of 1.2 after 48 h.

Content and type of sugars present in the extracts are determinant factors for the growth and viability of probiotic bacteria. Since no studies on prebiotic potential of *Ph. nameko* were found in the literature some pertinent observations with other mushroom species are described. For example, Synytsya et al. (2009) evaluated different extracts of *Pleurotus ostreatus* and *Pleurotus eryngii* for their potential prebiotic activity. According to these authors, two types of glucans and proteoglucan complexes from *Pleurotus* can be used as synbiotics with selected probiotic strains. Li and Shah (2016) recently reported potential bifidogenic effects of polysaccharides from *Pleurotus eryngii*. According to our results, the majority of the extracts obtained from *Ph. nameko* possess compounds that can be metabolized by *L. acidophilus* La-5 and *B. animalis* BB12. Higher prebiotic potential was observed for HWE, UAE, Viscozyme and Cellulase extracts. According to FTIR-ATR spectra of *Ph. nameko* (Rodrigues et al., 2015a), the presence of β-glucans, α-glucans and glucan-protein complexes is among the main representative polysaccharides in the *Ph. nameko* mushroom; their presence in the EA was also observed, as discussed later in this article. Given the reported results in terms of growth promotion of both probiotic bacteria and considering the prebiotic potential of polysaccharides and extracts from mushrooms reported by literature concerning other species, the extracts obtained from *Ph. nameko* by sustainable and food compatible extraction methods can be used as supplements to design novel foods with potential prebiotic properties.

All the extracts of *Ph. nameko* showed α-glucosidase inhibitory activity although to different extensions (Fig. 1c). The extracts with the highest inhibitory activity were the UAE (49%) and EAE with Cellulase (50%). To our knowledge, there are no reports on potential antidiabetic of water-based *Ph. nameko* extracts and therefore, only comparisons with other mushroom species and other extraction modes are possible. For example, Liu et al. (2012) evaluated the anti-hyperglycemic and antioxidant activities of five wild edible mushrooms from Southwest China. Based on their results, ethanolic and aqueous extracts showed anti-hyperglycemic activity where aqueous extract of *Catathelasma ventricosum* revealed the highest α-glucosidase inhibitory activity (IC_{50} of 2.74 μg/mL). Zhu et al. (2014a) reported over 40% of α-glucosidase inhibitory activity from oyster mushroom polysaccharides. Hsu, Hsu, Lin, Cheng, and Yang (2013) reported α-glucosidase inhibitory properties by intracellular polysaccharides from *Coriolus versicolor* which were related to the presence of α-(1,4) glycosidic linkages and total relative percentage of D-glucose and D-galactose in the polysaccharide structure, other than triterpenoids. In our study UAE and Cellulase extracts had the highest contents in total sugars (Table 1) which probably evidences the presence of polysaccharides with α-glucosidase inhibitory properties. The use of α-glucosidase inhibitors is an important strategy in the prevention (functional food) or management (therapeutic agent) of blood glucose levels in type 2 diabetic and borderline patients.

3.2. Chemical characterization of selected extracts of Ph. nameko

From the six different extracts, two were selected based on their major biological potential for further characterization. In Table 3a, a summary of the proximate composition and of the

Table 3

a) Basis of selection of two extracts of *Ph. nameko* considering the proximate composition and biological properties [antioxidant (ABTS, $HO^{-}\cdot$), prebiotic and antidiabetic activities); b) Elemental inorganic and organic composition, monosaccharides, uronic acids and amino-monosacharide composition as well as free amino acids composition in enzymatic extracts of *Ph. nameko*.

a)

	Extraction		Proximate characterization			Antioxidant Activity		Prebiotic activity		Antidiabetic activity
	Method	Yield	Nitrogen	Total Sugars	Total Phenolics	ABTS	$HO^{-}\cdot$	La-5	BB12	α-GIA
Ph. nameko	HWE		(+)	(+)	(-)	(+)	(+)	(++)	(++)	(+)
	UAE	(+)	(+)	(++)	(-)	(-)	(++)	(++)	(++)	(++)
	EAE_Cell	(+)	(-)	(++)	(-)	(-)	(-)	(++)	(++)	(++)
	EAE_Visc	(++)	(-)	(+)	(+)	(+)	(++)	(++)	(++)	(+)
	EAE_Alc	(+)	(++)	(-)	(+)	(++)	(+)	(++)	(+)	(-)
	EAE_Flav	(-)	(+)	(+)	(++)	(+)	(+)	(++)	(++)	(++)

b)

			Ph. nameko			
			EAE_Flav		EAE_Cell	
			($mg/g_{lyophilized\ extract}$)	Ratio[1]	($mg/g_{lyophilized\ extract}$)	Ratio[1]
Inorganic	Macro elements	K	126±1	3.4	98.0±1	2.6
		Na	20±0.4	-	29.7±0.9	-
		Ca	0.6±0.1	2.6	0.24±0.02	1.1
		Mg	6.3±0.2	4.0	5.5±0.2	3.5
		P	25.1±0.6	2.3	22.4±0.4	2.1
	Micro elements	Zn	0.36±0.02	2.9	0.311±0.004	2.6
		B	0.043±0.001	1.7	0.041±0.002	1.7
		Mn	0.120±0.004	6.8	0.108±0.002	6.1
		Fe	0.34±0.03	1.2	0.28±0.01	1
		Al	<LOD	-	<LOD	-
		Cu	0.086±0.002	3.1	0.073±0.002	2.7
		Ni	<LOD	-	<LOD	-
		Pb	0.008±0.001	-	0.006±0.001	-
Organic		%N	5.3	-	4.4	-
		%C	37.6	-	36.0	-
		%H	9.0	-	8.5	-
		%S	0.6	-	0.8	-

		Ph. nameko	
		EAE_ Flav	EAE_Cell
		($mg/g_{lyophilized\ extract}$)	($mg/g_{lyophilized\ extract}$)
Monosaccharides	DL-Glucose	28.3±0.4	31.1±0.3
	DL-Galactose	40.3±0.4	30.9±0.4
	DL-Mannose	16.2±0.2	17.9±0.3
	DL-Arabinose	0.49±0.01	0.46±0.01
	D-Xylose	0.29±0.01	0.21±0.1
	L-Rhamnose	<LOD	<LOD
	L-Fucose	<LOD	<LOD
Uronic acids	D-Glucuronic acid	<LOD	<LOD
	D-Galacturonic acid	<LOD	<LOD
Amino-mono.	D-glucosamine	3.7±0.1	4.5±0.1

	Ph. nameko	
	EAE_Flav	EAE_ Cell
	($g/100g_{protein}$)	($g/100g_{protein}$)
Aspartic acid	1.26±0.09	2.4±0.1
Glutamic acid	<LOD	1.02±0.09
Methionine	1.5±0.1	1.5±0.2
Serine	0.21±0.01	0.9±0.1
Glutamine	<LOD	0.25±0.06
Alanine	4.5±0.2	1.0±0.1
Leucine	0.25±0.02	<LOD
Threonine	1.2±0.1	<LOD
Valine	2.98±0.01	<LOD

Amino acids

Extraction yield (%): 54–61 (−); 62–69 (+); 70–77 (++).
Nitrogen ($mg/g_{lyoph\ extract}$): 40–43 (−); 44–47 (+); 48–52 (++).
Total sugars ($mg_{glucose\ equiv}/g_{lyoph\ extract}$): 376–459 (−); 460–543 (+); 544–628 (++).
Total phenolics ($\mu g_{cathecol\ equiv}/g_{lyoph\ extract}$): 144–156 (−); 157–169 (+); 170–182 (++).
ABTS (% scavenging activity): 37–40 (−); 41–43 (+); 44–47 (++).
$OH^{-}\cdot$ (% scavenging activity): 51–53 (−); 54–56 (+); 57–60 (++).
La5 [Log (cfu/mL)]: No increment of viable cells after 48 h in comparison to 0 h (−); No increment of viable cells after 48 h in comparison to 0 h but higher than values obtained with FOS (+); Similar or higher values of viable cells after 48 h in comparison to 0 h and higher than values obtained with FOS (++).
BB12 [Log (cfu/mL)]: No increment of viable cells after 48 h in comparison to 0 h (−); No increment of viable cells after 48 h in comparison to 0 h but higher than values obtained with FOS (+); Similar or higher values of viable cells after 48 h in comparison to 0 h and higher than values obtained with FOS (++).
α-Glucosidase inhibitory activity (% Inhibition): 14–25 (−); 26–37 (+); 38–50 (++).
[1] Ratio = Content in lyophilized extract/Content in dry mushroom; Values of organic elements are presented as average of triplicate samples.

biological properties observed in the extracts are given. Taking into account these results, selection fell on the EA obtained with Cellulase and with Flavourzyme both highlighted in Table 3a. The best potential in terms of antidiabetic and prebiotic activities of both EA was the main reason for their selection. The selection of the EA obtained with Flavourzyme over extract obtained with UAE was mainly due to its potential total antioxidant capacity despite its lower extraction yields.

An increment of the majority of macro and micro inorganic elements was observed in both EA (Table 3b) in comparison to the corresponding dry mushroom (Rodrigues et al., 2015a). Not much difference however was observed among the inorganic elemental composition of the two EA. It is evident from Table 3b that K and P are the main macro elements present in both EA. The content of K is especially high in comparison to Na, an advantage from the nutritional point of view. Magnesium is the third major macro element found with similar levels in both extracts. Calcium in turn, was not significantly present in the two extracts; nevertheless the Flavourzyme extract contained three-fold higher amounts than the Cellulase extract. In comparison to the macro element composition of the dried mushrooms (Rodrigues et al., 2015a) significant increments between 2.1 and 4.0 were observed for the macro elements Mg, K and P (in descending order of magnitude) in both extracts. An important increment of 2.6 was also observed for Ca in the EA obtained with Flavourzyme. Overall, both EA seem to be good sources of these macro elements. In terms of micro elements, Mn, Zn and Cu are among those with increments between 2.6 and 6.8. Iron and Zn were the most abundant elements among the trace minerals in the dry mushrooms and in the corresponding EA. Zinc is involved in the structure and function of over 300 enzymes, especially in superoxide dismutase (Zheng et al., 2014).

The organic elemental contents were similar in both EA (Table 3b), although a slightly higher content in N, C and H was observed for the Flavourzyme extract which could be related to the higher nitrogen content found in this extract (Table 1); on the other hand, in the Cellulase extract it was registered a higher content in total sugars.

The monosaccharides were the major constituents found with hexoses predominating over pentoses with undetectable levels of uronic acids (Table 3b). The Flavourzyme extract was characterized by slightly higher contents of monosaccharides with predominance of galactose (40%), glucose (28%) and mannose (16%). In comparison, the extract obtained with Cellulase possesses lower content of galactose (31%), higher content of glucosamine (4.5%) but similar content in terms of glucose (31%) and mannose (18%). According to Zheng et al. (2014) a zinc polysaccharide of *Ph. nameko* was composed of glucose, mannose, galactose and

arabinose in a molar ratio of 33.0:4.5:4.3:1.0. A different molar ratio was in turn reported by Li, Zhang, and Ma (2010) for a polysaccharide from *Ph. nameko* with hypolipidemic effect composed mainly of mannose, glucose, galactose, arabinose and xylose (1:8.4:13.6:29.6:6.2).

According to Wasser (2002) mushroom polysaccharides are present mostly as glucans with different types of glycosidic linkages, such as (1→3), (1→6)-β-glucans and (1→3)-α-glucans but also as heteroglucans; In this case, side chains of the polysaccharides contain glucuronic acid, xylose, galactose, mannose, arabinose, or ribose as a main component or in different combinations. Since no glucuronic acid was detected in the two EA of *Ph. nameko*, it could indicate absence of heteroglucans.

Glucosamine present in relatively low amounts (3.7–4.5 mg/$g_{\text{lyophilized extract}}$) in both EA was also observed in crude polysaccharides and in their fractions of *L. edodes*, *G. frondosa* and *T. versicolor* (Siu, Chen, & Wu, 2014). Chitin, a polymer of N-acetyl-glucosamine alongside with β-glucans, is the primary structural material in mushrooms and has been shown to be of value as dietary fibre which can be hydrolysed to glucosamine (Sonawane, Sathe, More, Jadhav, & Kadam, 2013).

Higher content and diversity of free amino acids were observed in EA obtained by Flavourzyme (Table 3b), a multi-enzyme complex with *endo*-protease and *exo*-peptidase, resulted in a higher nitrogen content (Table 1) and higher content of free amino acids (11.9 g/100g_{protein}) than the Cellulase action (7.1 g/100g_{protein}). Out of the different amino acids which constitute the nitrogen moiety of the extract obtained with Flavourzyme, the major amino acids appeared to be alanine (38%), followed by valine (25%), methionine (13%), aspartic acid (11%) and threonine (10%). On the other hand, aspartic acid (34%), methionine (21%), glutamic acid (15%), threonine (14%) and serine (13%) were the major amino acids in the extract obtained with Cellulase. Noticeably, the essential amino acids methione, valine, threonine and leucine make up 50% of the amino acid composition of the Flavourzyme extract. Of particular interest is the fact that no aromatic amino acids, phenylalanine and tyrosine, were detected in either of the *Ph. nameko* extracts.

3.3. Structural characterization of selected extracts of Ph. nameko

FTIR-ATR spectra of the two EA as well as of *Ph. nameko* mushroom are depicted in Fig. 2. Some differences, in terms of qualitative and absorbance intensity, are observed between spectra of the mushroom and respective EA; four specific regions (4000–1800 cm^{-1}, 1800–1500 cm^{-1}, 1500–950 cm^{-1} and 950–750 cm^{-1}) are observable in the three spectra:

(i) In the region between 4000 and 1800 cm^{-1}, the prominent band centred around 3300 cm^{-1} may be assigned to O—H stretching vibrations of glyosidic structures. According to Klaus et al. (2015) characteristic N—H vibration at 3400 cm^{-1} could be overlapped by O—H stretch vibration at 3000–3500 cm^{-1} of inter- and intra-hydrogen bonds that are present in polysaccharides. The bands around 2900–2880 cm^{-1}, in turn assigned to CH_2 and CH_3 stretching of fatty acids from the cell wall (Zheng et al., 2014; Zhao et al., 2006a,b), are more defined in the mushroom spectrum than in the respective extracts spectra, in particular for the 2900–2880 cm^{-1}. This fact could result from loss of fatty acids as consequence of the extraction process;

(ii) In the region between 1800 and 1500 cm^{-1}, two major bands around 1600 and 1500 cm^{-1} assigned to amide I and amide II of proteins (Klaus et al., 2015; Zhao et al., 2006a, b) are visible in mushroom spectra but not in the respective EA. In the EA only the band around 1580 cm^{-1} is observable being much more pronounced for the Flavourzyme extract spectrum than for its Cellulase counterpart; such observation may be related to the role of the Flavourzyme, with its *endo*-proteases and *exo*-peptidases, on *Ph. nameko* proteins which effectively resulted in a higher nitrogen content (Table 1), higher free amino acids content and antioxidant potential comparatively to the Cellulase extract (Table 3b). In addition an absorbance band at 1740 cm^{-1} corresponding to carbonyl stretching vibration of alkyl-esters (Zhao et al., 2006a,b) is present in the *Ph. nameko* and its Flavourzyme extract spectra but not in its Cellulase extract spectrum;

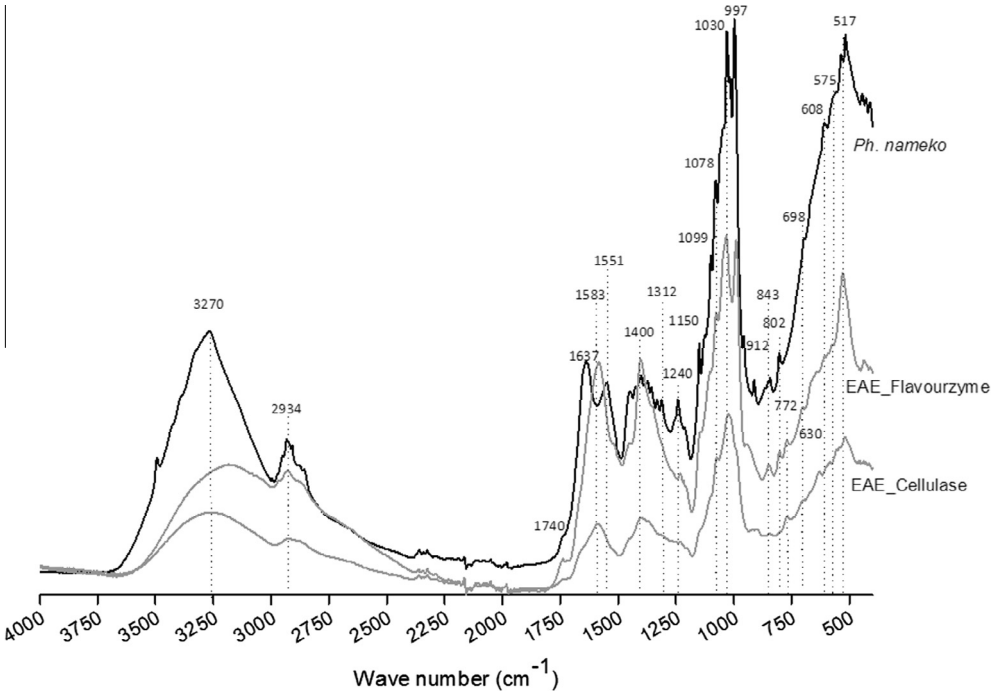

Fig. 2. FTIR-ATR spectra of the cultivated mushroom *Pholiota nameko* (—) and of its enzymatic extracts obtained by Flavourzyme (—) and Cellulase (—).

(iii) In the region 1500–950 cm^{-1} which is associated with vibrations of proteins, lipids and polysaccharides, absorption bands at 1400 and 1240 cm^{-1} are visible both in the mushroom and in its extracts spectra. These bands have been associated to protein structures (Gonzaga, Ricardo, Heatley, & Soares, 2005) which could be due to the presence of glucan-protein complex and role of the Flavourzyme. However according to Zheng et al. (2014), the absorption band around 1400 cm^{-1} could also suggest the occurrence of uronic acids (O—C=O bending) which were, however, not detected in both EA (Table 3b). Other authors in turn indicate that absorption bands between 1410 and 1310 cm^{-1} could be assigned to OH groups of phenolic compounds (Klaus et al., 2015). Indeed, the Flavourzyme extract was characterized by higher contents of nitrogen and total phenolics but lower content of total sugars in comparison to the Cellulase extract counterpart (Table 1). The presence of polysaccharides in both EA could be confirmed by the presence of bands around 1150, 1070 and 1040 cm^{-1} in Fig. 2, which could be responsible for both prebiotic and antidiabetic activities observed (Fig. 1c and Table 2). The band 1040 cm^{-1} has been assigned to C—O stretching, 1070 cm^{-1} to the anomeric C_1H group vibration and 1150 cm^{-1} to C—O—C stretching of glycosidic structures (Gonzaga et al., 2005; Klaus et al., 2015). According to Zheng et al. (2014), the strong characteristic absorption at 1200–1000 cm^{-1} is ascribed to sugar ring vibrations overlapping with stretching vibrations of C—OH side groups and the C—O—C glycosidic bonds vibration. An higher band intensity at 1200–1000 cm^{-1} is observable in the Flavourzyme extract than in the Cellulase extract. This could be related to the role of Cellulase on degradation of polysaccharides which could be related to the observed α-glucosidase inhibition (Fig. 1) and prebiotic effect on both probiotic bacteria (Table 2). In fact, the Cellulase extract contained higher sugar content than the Flavourzyme extract (Table 1) but a slightly lower content of monosaccharides and amino-monosaccharides (Table 3b);

(iv) In the 950–750 cm^{-1} region which has been associated with identification of anomeric configuration of polysaccharides, in particular around 890 cm^{-1} assigned to β-glycosides and 860–810 cm^{-1} to α-glycosides (Klaus et al., 2015; Zhao et al., 2006a,b; Zheng et al., 2014), those bands are clearly present in the spectrum of the Flavourzyme extract but

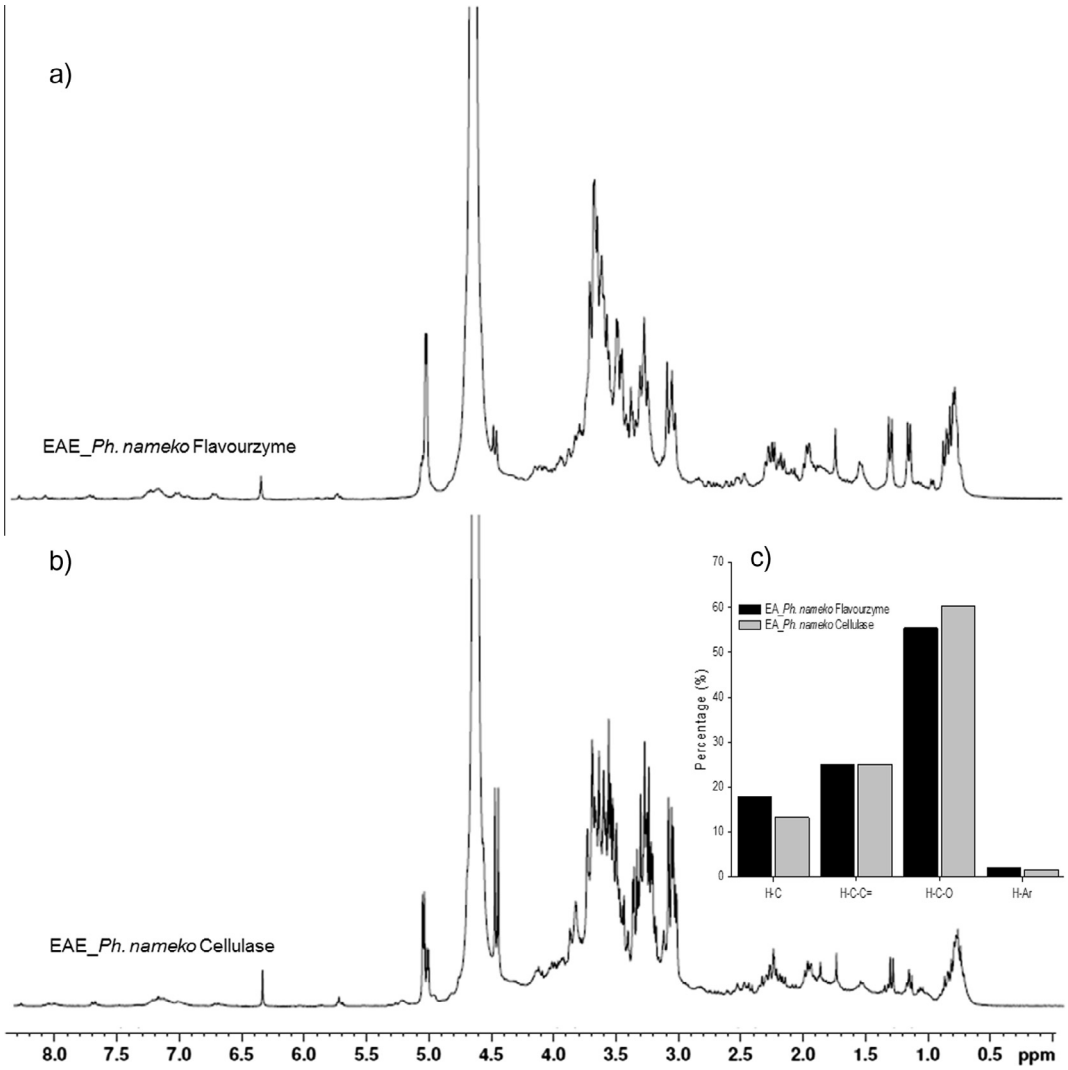

Fig. 3. ^{1}H NMR spectra of enzymatic extracts from *Pholiota nameko* obtained by Flavourzyme (a) and Cellulase (b) where peak at 4.7 ppm indicates the water signal and, relative abundance of each type of protons (c) estimated as the partial integrals of the spectra: H—C: purely alkylic hydrogen atoms; H—C—C=: hydrogen atoms in alpha position to C=C or C=O groups; H—C—O: aliphatic C—H directly bound to an oxygen atom; H—Ar: aromatic hydrogen atoms.

not so intense in the spectrum of the Cellulase extract in accordance with the bands pattern in the region 1200–1000 cm^{-1} described above.

The presence of β-glucans, α-glucans and glucan-protein complexes is evident in the *Ph. nameko* mushroom, which is in accordance to Rodrigues et al. (2015a), as well as in the EA which could be, at least in part, responsible for the prebiotic activity observed (Tables 2 and 3a).

The ^{1}H NMR spectra of both EA are quite similar (Fig. 3a–b). The spectra exhibit some distinct peaks overlaying much broader bands, as would be expected from the NMR spectra of complex mixtures of organic compounds (Santos, Santos, & Duarte, 2012). Despite the large variety of overlapping resonances, each ^{1}H NMR spectrum was investigated on the basis of the chemical shift assignments described in the literature for organic compounds (Clarke & Haselden, 2008; Lopes, Matos, Silva, Duarte, & Duarte, 2015). In accordance, four main regions of chemical shifts were considered in each spectrum:

1) δ_H = 0.6–1.8 ppm: aliphatic protons, H—C; —CH > —CH_2 > —CH_3;
2) δ_H = 1.8–3.2 ppm: protons bound to carbon atoms in alpha position to unsaturated groups in allylic (H—Cα—C=), carbonyl or imino (H—Cα—C=O or H—Cα—C=N) groups, and protons in secondary and tertiary amines (H—C=NR_2 and NR_3);
3) δ_H = 3.2–4.1 ppm: aliphatic protons on carbon atoms singly bound to oxygen atoms (H—C—O: H—C—O—CO—R > H—C—OH or H—C—O—C) in alcohols, polyols, ethers and esters;
4) δ_H = 6.5–8.5 ppm (aromatic protons).

For a further understanding of the ^{1}H NMR data, a quantitative integration of each spectral region was performed in order to assess the abundance of each of the different types of protons in the different extracts and depicted in Fig. 3c. A fifth region (δ_H = 4.1–6.0 ppm) corresponding to anomeric protons of glycosidic structures (Gonzaga et al., 2005) was also considered but not integrated due to the wide and intense peak at 4.7 ppm associated with the water signal.

In accordance to the spectra, the relative abundance of each type of protons is, in general, relatively similar among the two EA but some points are worthy of being highlighted. The extract obtained with Cellulase revealed slightly higher values for the H—C—O group which is probably related to the slightly higher content of total sugars and of some monosaccharides in comparison to that obtained with Flavourzyme (Tables 1 and 3b). In addition, slightly higher content of purely alkylic hydrogen atoms (H—C) is observed for the extract obtained with Flavourzyme. Signals assigned to terminal methyl hydrogens H_3C—C ($\delta_H \approx 0.9$ ppm) and to polymethylene chains $(CH_2)_n$ ($\delta_H \approx 1.3$ ppm) are seen in both spectra. No differences are observed for the other two groups of protons namely for the group of aromatic protons (H—Ar) and for the group of hydrogen atoms in alpha position to unsaturated groups in allylic (H—Cα—C=), carbonyl or imino (H—Cα—C=O or H—Cα—C=N) groups.

In the anomeric spectral region, a doublet at $\delta_H \approx 4.5$ ppm and at $\sim\delta_H \approx 5.0$ ppm is observed in both extracts (Fig. 3a–b) with particular intensity in the spectrum of the extract obtained with Cellulase, which could be assigned to β and α configurations, respectively. These data are in agreement with FTIR-ATR analysis which assigned the presence of α- and β-glycosides and to data reported by Gonzaga et al. (2005) for isolated polysaccharides from *Agaricus blazei* mushroom. No data for *Ph. nameko* was found in the literature.

4. Conclusions

Enzymatic and ultrasound assisted extractions are the important tools to obtain extracts from standard cultivated *Ph. nameko* with potential biological properties. Hydrolytic enzymes were able to improve the extraction yield with carbohydrases extracting more compounds from the cell structure of the mushrooms. However taking into consideration the general results achieved it becomes difficult to pinpoint the most efficient extraction method with the highest biological potential since it depends on the extraction mode considering the duality: extraction yield vs chemical/biological properties and on the type of analysis made. In this perspective, all *Ph. nameko* extracts presented added value in particular due to the interesting prebiotic potential. In addition, higher α-glucosidase inhibitory activity was observed in UAE, Cellulase and Flavourzyme extracts (in descending order of magnitude) and consequently an interesting antidiabetic potential is proposed. In terms of antioxidant capacity, no strong activity was observed. Therefore, conjugating extraction yield with prebiotic effect and α-glucosidase inhibitory activity, EA are of interest where probably the polysaccharides play the major role. In general, enzymatic extraction enabled the recovery of important compounds with nutritional or biological significance. Important concentration of almost all macro and micro elements in comparison to the dry mushroom, in particular for Mg, K and P was observed in both selected EA. Content and profile of monosaccharides varied slightly among the Flavourzyme and Cellulase extracts with predominance of glucose, galactose and mannose with no detection of uronic acids. Higher content and diversity of free amino acids were observed in Flavourzyme extract probably due to *endo*-protease and *exo*-peptidase action. According to chemical and structural analysis by FTIR-ATR and ^{1}H NMR, both EA, with prebiotic and antidiabetic potential, are composed by important polysaccharide structures, confirming the presence of α and β-glyosidic structures as well as the presence of α-glucans, β-glucans and glucan-protein complexes among the main representative polysaccharides. Therefore EA, from *Ph. nameko* have potential prebiotic and antidiabetic activities, which can be used as supplements for the design of novel functional foods.

Conflict of interest

The authors declare no conflict of interest.

Note

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

Acknowledgments

This work was supported by Portuguese Science Foundation (FCT – Fundação para a Ciência e Tecnologia) through individual research grants references SFRH/BPD/73781/2010 and SFRH/BD/77647/2011 under QREN – POPH funds, co-financed by the European Social Fund and Portuguese National Funds from MCTES. Thanks are due, for the financial support to CESAM (UID/AMB/50017), to FCT/MEC through national funds, and the co-funding by the FEDER (Fundo Europeu de Desenvolvimento Regional), within the PT2020 Partnership Agreement and Compete 2020. For CBQF, this work was supported by National Funds from FCT through project UID/Multi/50016/2013.

References

Bhakta, M., & Kumar, P. (2013). Mushroom polysaccharides as a potential prebiotics. *International Journal of Health Sciences and Research, 3*, 77–84.

Cheung, Y.-C., Siu, K.-C., Liu, Y.-S., & Wu, J.-Y. (2012). Molecular properties and antioxidant activities of polysaccharide–protein complexes from selected mushrooms by ultrasound-assisted extraction. *Process Biochemistry, 47*, 892–895.

Clarke, C. J., & Haselden, J. N. (2008). Metabolic profiling as a tool for understanding mechanisms of toxicity. *Toxicologic Pathology, 36*, 140–147.

Cravotto, G., Boffa, L., Mantegna, S., Perego, P., Avogadro, M., & Cintas, P. (2008). Improved extraction of vegetable oils under high-intensity ultrasound and/or microwaves. *Ultrasonics Sonochemistry, 15*, 898–902.

Gonzaga, M. L. C., Ricardo, N. M. P. S., Heatley, F., & Soares, S. A. (2005). Isolation and characterization of polysaccharides from *Agaricus blazei* Murill. *Carbohydrate Polymers, 60*, 43–49.

Heo, S.-J., Jeon, Y.-J., Lee, J., Kim, H. T., & Lee, K.-W. (2003). Antioxidant effect of enzymatic hydrolyzate from a kelp, *Ecklonia cava*. *Algae, 18*, 341–347.

Hsu, W.-K., Hsu, T.-H., Lin, F.-Y., Cheng, Y.-K., & Yang, J. P.-W. (2013). Separation, purification, and α-glucosidase inhibition of polysaccharides from *Coriolus versicolor* LH1 mycelia. *Carbohydrate Polymers, 92*, 297–306.

Ji, H., Zhang, L., Zhang, H., Li, G., & Yang, M. (2012). Antioxidant activities of extracts from *Pholiota nameko*. *Advanced Materials Research, 343–344*, 457–462.

Klaus, A., Kozarski, M., Vunduk, J., Todorovic, N., Jakovljevic, D., Zizak, Z., ... Van Griensven, L. J. L. D. (2015). Biological potential of extracts of the wild edible Basidiomycete mushroom *Grifola frondosa*. *Food Research International, 67*, 272–283.

Li, H., Lu, X., Zhang, S., Lu, M., & Liu, H. (2008). Anti-inflammatory activity of polysaccharide from *Pholiota nameko*. *Biochemistry, 73*, 669–675.

Li, S., & Shah, N. P. (2016). Characterization, antioxidative and bifidogenic effects of polysaccharides from *Pleurotus eryngii* after heat treatments. *Food Chemistry, 197*, 240–249.

Li, H., Tao, Y., Zhao, P., Huai, L., Zhi Liu, J., ... Xu, Y. (2015). Effects of *Pholiota nameko* polysaccharide on NF-κB path way of murine bone marrow-derived dendritic cells. *International Journal of Biological Macromolecules, 77*, 120–130.

Li, H., Zhang, M., & Ma, G. (2010). Hypolipidemic effect of the polysaccharide from *Pholiota nameko*. *Nutrition, 26*, 556–562.

Liu, Y.-T., Sun, J., Luo, Z.-Y., Rao, S.-Q., Su, Y.-J., Xu, R.-R., & Yang, Y. J. (2012). Chemical composition of five wild edible mushrooms collected from Southwest China and their antihyperglycemic and antioxidant activity. *Food and Chemical Toxicology, 50*, 1238–1244.

Lopes, S. P., Matos, J. T. V., Silva, A. M. S., Duarte, A. C., & Duarte, R. M. B. O. (2015). ^{1}H NMR studies of water- and alkaline-soluble soluble organic matter from fine urban atmospheric aerosols. *Atmospheric Environment, 119*, 374–380.

Mazzutti, S., Ferreira, S. R. S., Riehl, C. A. S., Smania, A., Jr., Smania, F. A., & Martínez, J. (2012). Supercritical fluid extraction of *Agaricus brasiliensis*: Antioxidant and antimicrobial activities. *Journal of Supercritical Fluids, 70*, 48–56.

Moro, C., Palacios, I., Lozano, M., D'Arrigo, M., Guillamón, E., Villares, A., ... García-Lafuente, A. (2012). Anti-inflammatory activity of methanolic extracts from edible mushrooms in LPS activated RAW 264.7 macrophages. *Food Chemistry, 130*, 350–355.

Palanisamy, M., Aldars-García, L., Gil-Ramírez, A., Ruiz-Rodríguez, A., Marín, F. R., Reglero, G., & Soler-Rivas, C. (2014). Pressurized water extraction of β-glucan enriched fractions with bile acids-binding capacities obtained from edible mushrooms. *Biotechnology Progress, 30*, 391–400.

Proestos, C., Loukatos, P., & Komaitis, M. (2008). Determination of biogenic amines in wines by HPLC with precolumn dansylation and fluorimetric detection. *Food Chemistry, 106*, 1218–1224.

Puri, M., Sharma, D., & Barrow, C. J. (2012). Enzyme-assisted extraction of bioactives from plants. *Trends in Biotechnology, 30*, 37–44.

Quian, L., Zhang, Y., & Liu, F. (2015). Purification and characterization of a ~43 kDa antioxidant protein with antitumor activity from *Pholiota nameko*. *Journal of Science and Food Agriculture, 96*, 1044–1052.

Rodrigues, D., Freitas, A. C., Rocha-Santos, T. A. P., Vasconcelos, M. W., Roriz, M., Rodríguez-Alcalá, L. M., ... Duarte, A. C. (2015a). Chemical composition and nutritive value of *Pleurotus citrinopileatus* var *cornucopiae*; *P. eryngii*, *P. salmoneo stramineus, Pholiota nameko* and *Hericium erinaceus*. *Journal of Food Science and Technology, 52*, 6927–6939.

Rodrigues, D., Sousa, S., Silva, A. G., Amorim, M., Pereira, L., Rocha-Santos, T. A. P., ... Freitas, A. C. (2015b). Impact of enzyme- and ultrasound-assisted extraction methods on biological properties of red, brown, and green seaweeds from the central west coast of Portugal. *Journal of Agriculture and Food Chemistry, 63*, 3177–3188.

Ruiz-Herrera, J. (1991). *Fungal cell wall: Structure, synthesis, and assembly*. USA: CRC Press.

Santos, P. S. M., Santos, E. B. H., & Duarte, A. C. (2012). First spectroscopic study on the structural features of dissolved organic matter isolated from rainwater in different seasons. *Science of The Total Environment, 426*, 172–179.

Seephonkai, P., Samchai, S., Thongsom, A., Sunaart, S., Kiemsanmuang, B., & Chakuton, K. (2012). DPPH radical scavenging activity and total phenolics of *Phellinus* mushroom extracts collected from northeast of Thailand. *Chinese Journal of Natural Medicines, 9*, 441–445.

Siu, K., Chen, X., & Wu, J. (2014). Constituents actually responsible for the antioxidants activities of crude polysaccharides isolated from mushrooms. *Journal of Functional Foods, 11*, 548–556.

Sonawane, A. A., Sathe, S. B., More, S. H., Jadhav, A. P., & Kadam, V. J. (2013). Glucosamine: A boon to osteoarthritis. *Indo American Journal of Pharmaceutical Research, 3*, 7122–7128.

Synytsya, A., Mičkova, K., Synytsya, A., Jablonský, I., Spěvaček, J., Erban, V., ... Čopíková, J. (2009). Glucans from fruit bodies of cultivated mushrooms *Pleurotus ostreatus* and *Pleurotus eryngii*: Structure and potential prebiotic activity. *Carbohydrate Polymers, 76*, 548–556.

Tian, Y., Zeng, H., Xu, Z., Zheng, B., Lin, Y., Gan, C., & Lo, M. (2012). Ultrasonic-assisted extraction and antioxidant activity of polysaccharides recovered from white button mushroom (*Agaricus bisporus*). *Carbohydrate Polymers, 88*, 522–529.

Wasser, S. P. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology, 60*, 258–274.

Yin, X., You, Q., & Jiang, Z. (2011). Optimization of enzyme assisted extraction of polysaccharides from *Tricholoma matsutake* by response surface methodology. *Carbohydrate Polymers, 86*, 1358–1364.

Zhang, J., Xu, N., Wang, G., Zhao, H., Lin, L., & Jia, L. (2015). In vitro and in vivo antioxidant effects of polysaccharides from Nameko medicinal mushroom, *Pholiota nameko* SW-01 (Higher Basidiomycetes). *International Journal of Medicinal Mushrooms, 17*, 671–680.

Zhao, D., Liu, G., Song, D., Liu, J., Zhou, Y., Ou, J., & Sun, S. (2006a). Fourier transform infrared spectroscopic study of truffles. In *Proc. SPIE 6026, ICO20: Biomedical optics*. . 60260H, China.

Zhao, D., Liu, G., Song, D., Liu, J.-H., Zhou, Y., Ou, J., & Sun, S. (2006b). Identification of Amanita mushrooms by Fourier transform infrared spectroscopy. In *Proceedings of SPIE 6047, Fourth international conference on photonics and imaging in biology and medicine*. . 60471V, China.

Zheng, L., Zhai, G., Zhang, J., Wang, L., Ma, Z., Jia, M., & Lia, L. (2014). Antihyperlipidemic and hepatoprotective activities of mycelia zinc polysaccharide from *Pholiota nameko*. *International Journal of Biological Macromolecules, 70*, 523–529.

Zhu, Y., Li, Q., Mao, G., Zou, Y., Feng, W., Zheng, D., ... Wu, X. (2014b). Optimization of enzyme-assisted extraction and characterization of polysaccharides from *Hericium erinaceus*. *Carbohydrate Polymers, 101*, 606–613.

Zhu, Z.-Y., Zhang, J.-Y., Chen, L.-J., Liu, X.-C., Liu, Y., Wang, W. X., & Zhang, Y. M. (2014a). Comparative evaluation of polysaccharides isolated from *Astragalus*, oyster mushroom, and yacon as inhibitors of α-glucosidase. *Chinese Journal of Natural Medicines, 12*, 290–293.