**Testing the effect of combining innovative extraction technologies on the biological activities
of obtained β -glucan-enriched fractions from *Lentinula edodes***

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extraction; hypocholesterolemic activity; immunomodulatory activity.

Abbreviations: HWE (hot water extraction), SPE (steam pressurized extraction), UAE
(ultrasound-assisted extraction), SWE (subcritical water extraction), SFE (supercritical fluid
extraction), MAE (microwave-assisted extraction), HPSEC (high-performance size-exclusion
chromatography system), HMGGR (3-hydroxy-3-methylglutaryl coenzyme A reductase).

19 **Abstract**

20 Innovative technologies as ultrasound-assisted extraction (UAE) (550W, 60% amplitude,
21 50°C) or subcritical water extraction (SWE) (200°C, 11.7 MPa) were more effective than hot water
22 extractions to obtain β -glucan-enriched fractions from shiitake mushrooms. UAE required longer
23 extraction time (60 min) than SWE (15 min). Combination of UAE+SWE or pre-treatment of the
24 raw material with supercritical CO₂ (SFE) (40°C, 35 MPa, 3h) before both extractions yielded
25 extracts containing larger β -glucan concentrations. Fluorimetric/colorimetric determinations
26 indicated that obtained fractions contained (1 $\rightarrow$ 3)- and (1 $\rightarrow$ 3),(1 $\rightarrow$ 6)- β -glucans. NMR confirmed
27 their presence as well as (1 $\rightarrow$ 3)- α -glucans and heteropolymers including mannose and galactose.
28 SWE (15 min), SFE+SWE or UAE+SWE extracts showed larger glucose levels and lower mannose
29 and galactose residues than the other extractions suggesting certain extraction specificity towards β -
30 glucans. They also included more chitin-derivatives than UAE. The extracts obtained after
31 combination of technologies partially retained their immunomodulatory properties but they showed
32 high hypocholesterolemic activities according to *in vitro* studies.

1. Introduction

Lentinula edodes is an Asian edible mushroom consumed worldwide and commonly known as shiitake mushroom. Traditionally, it was included in popular remedies to prevent diseases or to promote health and wellbeing and, in the last decades, scientific studies confirmed that indeed, the mushroom contains many bioactive compounds that might reduce the risk of harmful disorders such as cancer, cardio- or cerebrovascular diseases etc. (Khan, Gani, Khanday, & Massodi, 2018). Fungal β -glucans are compounds thoroughly investigated because they are considered as dietary fiber (together with chitins) with anti-inflammatory, immunomodulatory, antioxidant, anti-tumoral, hypocholesterolemic, antimicrobial activities, etc. (Abreu et al., 2019; Bai et al., 2019; Caz et al., 2016; Friedman, 2016; Khan et al., 2018; Zhang, Li, Wang, Zhang, & Cheung, 2011) and therefore, they might be used to design novel functional foods.

Mushroom β -glucans are structurally different than those from plants or bacteria since they have a main chain of β -D-glucose units (1 $\rightarrow$ 3)-linked with different branching usually at O-6 position by β -D-glucose units or other oligosaccharides. Their branching degree determines the tertiary structure of the glucan: (1 $\rightarrow$ 3)- β -glucans with few or no branches mainly present a single linear structure while highly branched (1 $\rightarrow$ 3),(1 $\rightarrow$ 6)- β -glucans show a triple helix conformation (Nitschke et al., 2011). In shiitake mushrooms, lentinan is the major (1 $\rightarrow$ 3),(1 $\rightarrow$ 6)- β -glucan and showed many beneficial properties for human health (Kupfahl, Geginat, & Hof, 2006; Suzuki, Takatsuki, Maeda, Hamuro, & Chihara, 1994). Since the integrity of the molecules is important to keep many of their beneficial properties, the extraction methods utilized to generate β -glucan-enriched extracts should be carefully selected because they might modify their molecular weight or tridimensional structure (Benito-Roman, Martin-Cortes, Cocero, & Alonso, 2016b).

Although some fungal polysaccharides are polar and easily extractable with water, many β -glucans require aggressive treatments such as hot water or alkaline solutions and others remain in the insoluble residue (Morales, Smiderle, Piris, Soler-Rivas, & Prodanov, 2019; Ruthes, Smiderle, & Iacomini, 2015). Moreover, chitins are water insoluble polymers and only low molecular weight derivatives or degradation products could be extracted using conventional hot water procedures

(Morales, Piris, Ruiz-Rodriguez, Prodanov, & Soler-Rivas, 2018a; Morales et al., 2019; Ruthes et al., 2015). To obtain chitinous materials, more drastic treatments are required to induce dissolving of other components in alkaline or acid solutions and the precipitation of chitin-enriched fractions (Wu, Zivanovic, Draughon, & Sams, 2004; Wu, Zivanovic, Draughon, Conway, & Sams, 2005). Novel advanced technologies such as ultrasound-assisted extractions (UAE) or subcritical water extraction (SWE) facilitate the extraction of these and other polysaccharides using water as non-pollutant solvent (Rosello-Soto et al., 2016, Maric et al., 2018). Ultrasound-assisted extractions generates ultrasonic waves provoking implosions of the generated cavitation bubbles, causing disruption of fungal cell walls. This treatment enhanced mass transfer and extraction yields (Chemat et al., 2016; Morales, Ruiz-Rodriguez, & Soler-Rivas, 2018b, Rosello-Soto et al., 2015) and it was successfully used to extract polysaccharides from several mushrooms (*Ganoderma lucidum*, *Agaricus bisporus*, *Boletus edulis*, etc.) and their by-products (Aguilo-Aguayo, Walton, Vinas, & Tiwari, 2017; Alzorqi, Sudheer, Lu, Manickam, 2017; Morales et al., 2018b). Pressurized liquid extractions constitute other interesting approach to enhance polysaccharide extraction, particularly SWE. When subcritical pressures are used, the solvent is heated above its boiling point keeping its liquid state and conferring it different properties than those showed at room temperature and atmospheric pressure. SWE modify dielectric constant of water and decrease its viscosity being able to solubilize non-polar compounds such as large size polysaccharides (when temperature is above 100 °C) (Li, Dobruchowska, Gerwig, Dijkhuizen, & Kamerling, 2013; Plaza & Turner, 2015; Smiderle et al., 2017; Morales et al., 2018b). Mushrooms polysaccharide-enriched extracts were obtained by SWE from many species such as *Agaricus bisporus*, *Pleurotus ostreatus*, *Ganoderma lucidum*, etc. (Palanisamy et al., 2014; Kodama et al., 2015; Smiderle et al., 2017).

Besides the previously mentioned, other green technologies were used to extract interesting compounds from edible mushrooms. Supercritical fluid extractions (SFE), particularly those using supercritical CO₂ as solvent, were mainly applied to selectively extract lipids from food matrices because of the low polarity of the solvent (Herrero, Cifuentes, & Ibáñez, 2006). Therefore, it might also be used to remove fat components from fungal extracts enhancing, in a subsequent step,

polysaccharide extraction, as described in previous works for *Antrodia camphorata* and *Agaricus blazei* mycelia (Chen et al., 2007; Ker et al., 2005). Combinations of different extraction technologies to obtain β -glucan-enriched extracts were also tested and higher yields than single extractions were usually noticed. For instance, in a complex extraction reactor, the use of pressurized hot water combined with sub/supercritical CO₂ increased 1.26 folds β -glucan yields obtained from *Ganoderma lucidum* than using only pressurized hot water (Benito-Roman, Alonso, Cocero, & Goto, 2016a). Moreover, ultrasonic/microwave assisted extractions (UMAE) were also more effective than hot water extractions to obtain bioactive polysaccharides from *Inonotus obliquus* (Chen, Huang, Li, Wang, & Tang, 2010) and combined with enzymes facilitated the extraction even more (Yin, Fan, Fan, Shi, & Gao, 2018).

Therefore, in this work, UAE and SWE were firstly carried out at different extraction times to define the most adequate to obtain β -glucan-enriched extracts from shiitake mushrooms. The extraction yields were compared with more conventional extractions. Afterwards, combinations of UAE and SWE as well as pre-treatments with SFE were investigated as innovative protocols to improve β -glucan extraction yields. The nature of bioactive polysaccharides from the extracts was determined by different methodologies (enzymatic and colorimetric methods, HPSEC, GC-MS, NMR) and their biological activities studied *in vitro* (hypocholesterolemic and immunomodulatory activities) to investigate whether the extraction procedure modify their beneficial properties.

2. Materials and Methods

2.1. Biological material

Powdered *Lentinula edodes* S. (Berkeley) fruiting bodies (particle size < 0.5 mm, moisture < 5%) were purchased from Glucanfeed S.L. (La Rioja, Spain) and stored in darkness at -20 °C until further use.

2.2. Reagents

Absolute ethanol and sulfuric acid (H₂SO₄) were obtained from Panreac and phenol, sodium borohydride (NaBH₄), sodium hydroxide pellets, glycine, D-glucose, glucosamine hydrochloride,

aniline blue diammonium salt 95%, hydrochloric acid 37%, acetylacetone, p-dimethylaminobenzaldehyde, trifluoroacetic acid, pyridine, acetic anhydride, copper(II) sulfate (CuSO₄), deuterated dimethylsulfoxide (Me₂SO-d₆), Congo Red, citric acid, RPMI 1640 medium and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich Quimica (Madrid, Spain). CO₂ was supplied by Air-Liquid, S.A. (Madrid, Spain), schizophyllan was obtained from Contipro Biotech (Dolni Dobrouc, Czech Republic) and synthetic soluble starch was acquired from Scharlab (Barcelona, Spain).

2.3. Supercritical CO₂ extraction pre-treatment (SFE)

Shiitake powder (253 g) was mixed with 1.9 kg of 6 mm diameter stainless steel spheres (ratio 1:1 (v/v) powder:spheres) in a 2 L extraction cell connected to a supercritical fluid extraction plant (model SF2000, TharTechnology, Pittsburgh, PA). Pressurized CO₂ was applied into the loaded cell at 35 MPa and 40 °C with a 3.6 kg/h recirculating flow during a total extraction time of 3 h as they were the conditions described as adequate for fungal lipids (Morales et al., 2017; Morales et al., 2018a). The lipophilic material solubilized in supercritical CO₂ was removed in separators (Garcia-Risco, Vicente, Reglero, & Fornari, 2011). After those 3h, the pressurized material remaining in the extraction cell (called SFE fraction) was separated from steel spheres by sieving in a sieve shaker (Cisa BA200 N, Barcelona, Spain) and stored at -20 °C until further use.

2.4. Ultrasound-assisted extractions (UAE)

Shiitake powder (1 g) was mixed in MilliQ water (100 mL) and submitted to sonication (Branson SFX550 Digital Sonifier 550W, Branson Ultrasonics, USA) using an ultrasonic probe (1/2'') and selecting 60% amplitude for sonication output. During processing, the temperature was maintained at 50 °C using a water bath with ice and a thermometer and samples were taken after 15, 30, 45 and 60 min extraction in duplicate. The obtained mixtures were submitted to vacuum filtration (through Whatman filter paper no. 1) to separate the soluble fraction from the insoluble residue. Afterwards, soluble fractions were lyophilized (UAE fractions) using a freeze-dryer

LyoBeta 15 (Telstar, Madrid, Spain) and stored at -20 °C until they were further processed to obtain polysaccharide-enriched extracts or submitted to subcritical water extractions.

The UAE fractions were mixed with water (50 g/L) and three volumes of ethanol to induce polysaccharide precipitation and left incubating overnight at 4 °C. The precipitates were collected by centrifugation (10 000 rpm, 10 min, 4 °C) in a Thermo Scientific Heraeus Multifuge (Thermo Fisher Scientific, Madrid, Spain) and freeze-dried. The obtained polysaccharide-enriched extracts (UAE extracts) were stored at -20°C until further analysis.

2.5. Subcritical water extractions (SWE)

Shiitake powder (0.5 g) was mixed with washed sea sand (Panreac, Barcelona, Spain) at ratio 1:8 (mushroom:sand, w/w) and placed in an extraction cell (11 mL) covered with cellulose filters (Dionex Corporation, USA) from an Accelerated Solvent Extractor (ASE) 350 (Dionex Corporation, USA). Extractions were carried out in duplicate with MilliQ water at 200 °C and 11.7 MPa but using different extraction times (15, 30, 45 and 60 min). Temperature was fixed at 200 °C as it was previously pointed as the ideal temperature to extract polysaccharides from shiitake mushrooms (Palanisamy et al., 2014). The obtained mixtures were vacuum filtrated and the soluble fractions were freeze-dried (SWE fractions) and used to generate polysaccharide-enriched extracts (SWE extracts) as above described.

2.6. Conventional extractions

Two different conventional extraction methods were followed to compare with the previously mentioned advanced technologies. One was a simple hot water extraction (HWE) and the other was a method frequently used to obtain polysaccharides using an autoclave (steam pressurized extraction, SPE).

Shiitake powder (0.5 g) was mixed in MilliQ water (50 mL), placed into a water bath at 100 °C and vigorously stirred during 15, 30, 45 and 60 min. Incubations were carried out in duplicate. Afterwards, the obtained mixtures were vacuum filtrated and the soluble fractions freeze-dried.

Obtained HWE fractions were later submitted to polysaccharide extraction as previously described to obtain hot water extracts (HWE extracts).

Similarly, the same shiitake mixture was heated at 120 °C for 20 min in an autoclave and cooled down to 4 °C following the method reported by Jeurink, Noguera, Savelkoul, & Wichers (2008). After the steam pressurized extraction (SPE), the mixture was also submitted to vacuum filtration and the soluble fraction freeze-dried (SPE fraction) and further processed to obtain SPE extracts as above indicated for HWE extracts.

2.7. Combined extractions

Sequential extractions were also tested as indicated in Figure 1 by combining SFE pre-treatment (35 MPa, 40°C, 3h) with ultrasound assisted extraction or subcritical water extraction. Thus, obtained SFE fraction was submitted to UAE (550W, 50°C, 60% amplitude) during 60 min as previously indicated for shiitake powder to generate a new SFE+UAE fraction that was further processed to obtain a polysaccharide-enriched extract (SFE+UAE extract) as also described for UAE extracts. Similarly, SFE fraction was submitted to SWE (200°C, 11.7 MPa, 15 min) obtaining a SFE+SWE fraction and a SFE+SWE polysaccharide-enriched extract (SFE+SWE extract). Moreover, the UAE fractions (550W, 50°C, 60% amplitude) obtained after 60 min sonication were also submitted to subcritical water extraction (200°C, 11.7 MPa) during 15 min to obtain the UAE+SWE fractions. Afterwards, polysaccharide-enriched extracts (UAE+SWE extracts) were also generated following the same procedure as previously described for UAE extracts. All these combined extracts were prepared in duplicate and stored as indicated for the simple extracts.

2.8. Determination of carbohydrates

Total carbohydrate content of shiitake, the extracted fractions and the obtained polysaccharide-enriched extracts was determined by the phenol-sulfuric acid method as described in Fox & Robyt (1991).

Total β -glucan concentration was determined in the polysaccharide-enriched extracts using a mushroom and yeast specific β -glucan determination kit (β -glucan Assay Kit Megazyme®, Megazyme, Wicklow, Ireland) following the instructions of the user's manual. Samples absorbance was measured using a Genesys 10 UV spectrophotometer (Thermo Fisher Scientific, Madrid, Spain).

The amount of (1 $\rightarrow$ 3)- β -glucans in the extracts was determined by the fluorimetric method described by Ko & Lin (2004) but including the modifications of Gil-Ramirez, Smiderle, Morales, Iacomini, & Soler-Rivas (2019) and Smiderle et al. (2017). The measurements were performed in a M200 Plate Reader (Tecan, Mannedorf, Switzerland) with excitation and emission wavelengths set at 398 and 502 nm respectively. The (1 $\rightarrow$ 3),(1 $\rightarrow$ 6)- β -glucan content of the extracts was analyzed following the colorimetric method (523 nm) described by Nitschke et al. (2011). Schizophyllan was used as standard for both determinations.

Chitin content was determined according to Smiderle et al. (2017). Briefly, precipitated extracts were hydrolyzed with 6 M HCl at 100 °C for 2 h. After the hydrolysis, samples were cooled down and adjusted to pH 10.0. Then, hydrolyzed samples (250 μ L) were treated as described by Rementeria et al. (1991) and absorbance was measured at 530 nm. A glucosamine hydrochloride standard curve was used for quantification.

2.9. GC-MS analyses

The monosaccharide composition of the polysaccharide-enriched extracts was determined by hydrolyzing the extracts (1 mg) with 2M trifluoroacetic acid at 100 °C for 8 h followed by evaporation to dryness. The dried samples were dissolved in distilled water (100 μ L) and NaBH₄ (1 mg) was added. Then, solution was kept at room temperature overnight to reduce aldose into alditols (Sasaki et al., 2008) and later, the samples were dried and the NaBH₄ excess was neutralized by adding acetic acid and then removed with methanol (twice) under a compressed air stream. Alditols acetylation was performed in pyridine-acetic anhydride (200 μ L; 1:1 v/v) for 30 min at 100 °C. Pyridine was removed by washing with 5% CuSO₄ solution and the resulting alditol

acetates were extracted with chloroform. The samples were injected into an SH-Rtx-5ms (30 m x 0.25 mm ID x 0.25 μ m thickness phase). The column was connected to a GC-2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a Combipal autosampler (AOC 5000) and coupled to a triple quadrupole mass spectrometer TQ 8040. The injector and ion source were held at 250 °C and helium at 1 mL/min was used as carrier gas. The oven temperature was programmed from 100 to 280 °C at 10 °C/min with a total analysis time of 30 min. The samples were prepared in hexane with 1 μ L being injected with a split ratio of 1:10. The mass spectrometer was operated in the full-scan mode over a mass range of m/z 50-500 before selective ion monitoring mode, both with electron ionization at 70 eV. Selective ion monitoring mode was used for quantification and *GCMS solution* software (Tokyo, Japan) was used for data analysis. The obtained monosaccharides were identified by their typical retention time compared to commercial available standards. Results were expressed as mol%, calculated according to Pettolino, Walsh, Fincher, & Bacic (2012).

2.10. HPSEC analyses

Polysaccharide-enriched extracts were injected into a high-performance size-exclusion chromatography system (HPSEC) (Waters, Massachussets, USA) coupled to refractive index detector (Waters). Four gel-permeation Ultrahydrogel columns in series with exclusion sizes of 7x10⁶, 4x10⁵, 8x10⁴ and 5x10³ Da were used. The eluent was 0.1 aqueous NaNO₂ containing 200 ppm aqueous NaN₃ at 0.6 mL/min. The extracts were dissolved in the eluent (1 mg/mL), filtered through a 0.22 μ m membrane and then, injected (100 μ L loop). Data were analyzed using Astra software version 4.70.

2.11. NMR analyses

NMR spectra (¹H, ¹³C and HSQC-DEPT) from precipitated extracts were obtained using a 400 MHz Bruker model Advance III spectrometer with a 5 mm inverse probe. The analyses were performed at 70 °C and the samples (30 mg) were dissolved in Me₂SO-*d*₆. Chemical shifts are expressed in ppm (δ) relative to Me₂SO-*d*₆ at 39.7 (¹³C) and 2.40 (¹H).

2.12. Determination of HMGCR inhibitory activity

The polysaccharide-enriched extracts were solubilized in water (50 mg/mL) and applied (20 μ L) into a 96-wells plate. Their inhibitory activity was measured using the commercial HMGCR (3-hydroxy-3-methylglutaryl coenzyme A reductase) activity assay (Sigma-Aldrich, Madrid, Spain) according to the manufacturer's instructions by monitoring their absorbance change (340 nm) at 37 °C using a 96-wells microplate reader BioTek Sinergy HT (BioTek, Winooski, USA). Pravastatin was used as a control for positive inhibition.

2.13. Macrophage cultures and immunomodulatory testing

The human monocyte THP-1 cell line was obtained from ATCC and cultured with supplemented RPMI 1640 medium (10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 2mM L-glutamine and 0.05 mM β -mercaptoethanol). For differentiation into macrophages, THP-1 cells were seeded (5×10^5 cells/mL) in 24 well-plate with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) and maintained for 48 h at 37 °C under 5% CO₂ in a humidified incubator

Firstly, the extracts cytotoxicity was evaluated in differentiated macrophages using 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) according to Mosmann (1983). Afterwards, the macrophages were washed with PBS and then replaced with serum-free medium containing LPS (0.05 μ g/mL) and subtoxic concentrations of the shiitake extracts. After 10 h of incubation, cells supernatants were collected and store at -20°C until use.

Pro-inflammatory cytokines TNF α (Tumour necrosis factor alpha), IL-1 β (Interleukin 1 beta) and IL-6 (Interleukin 6) were measured in the supernatants by BD Biosciences Human ELISA set (Aalst, Belgium) following the manufacturer's instructions. The quantification was calculated considering positive controls (cells stimulated with LPS) as a 100% cytokine secretion. The colour generated was determined by measuring the OD at 450 nm using a multiscanner autoreader (Sunrise, Tecan). Experiments were carried out in triplicate.

2.14. Statistical analyses

Differences were evaluated at 95% confidence level ($P \leq 0.05$) using a one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison test. Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA).

3. Results and Discussion

3.1. UAE and SWE as individual extraction methodologies

Polysaccharide extractions using ultrasounds or subcritical water were carried out at different extraction times and compared with conventional methodologies such as hot water extractions (HWE). When the obtained yields were evaluated (Table 1), results indicated that SWEs extracted more material from shiitake powder than UAEs, almost 2 fold more than HWEs at any extraction time and also more than a commonly utilized method to isolate polysaccharides using water at 120°C for 20 min (SPE) (Jeurink et al., 2008). These results might suggest that the high temperature of the pressurized water (that might have easily penetrated in the hyphae because it still maintains its liquid status) was more effective to dissolve and extract fungal compounds than other methods using lower temperatures such as 120, 100 or 50°C. Similarly, the propagation of ultrasonic waves might have also provided a greater water penetration into the fungal hyphae than more conventional methodologies using higher temperatures.

HWE yields were higher than reported in previous studies where extractions were carried out for 60 min at 98 °C (Morales et al., 2018a; Morales et al., 2019). However, the value obtained using the SPE method was in the range of previous works (Jeurink et al., 2008). Worth to notice was the decrease observed on SWEs carried out for more than 45 min. It might indicate clogging of the cellulose filter due to the large amount of extracted material. Therefore, if long extraction periods are required, extraction cycles are encouraged since similar studies but using extraction cycles (200 °C, 5 cycles of 5 min) yielded slightly higher values (Palanisamy et al., 2014).

The polysaccharides amount detected in UAE obtained fractions (Table 1) were in concordance with previous works where 9.75% polysaccharides were recorded from *L. edodes* after 21 min extraction (Zhao et al., 2018). Similarly, the SWE fractions obtained after 15 min extraction showed

a slightly higher polysaccharide content than noticed by Zhang, Yang, Lin, Zhao, & Wang (2019) but because only lower temperatures were tested (11.35 – 12.09%).

After extraction, the obtained fractions were submitted to precipitation to generate polysaccharide- enriched extracts (Figure 1). Results indicated that the HWE fractions contained less material susceptible of ethanol precipitation compared with UAE fractions and particularly the SWE fraction obtained after 15 min extraction. This fraction contained the highest polysaccharide levels yielding 15.5 g/100g SWE fraction, levels that were higher than those obtained using other advanced technologies such as UAE after 60 min (10.1 g/100g UAE fraction) or *e.g.* microwave-assisted extraction (MAE) (10.5 g/100 g MAE fraction) (Gil-Ramirez et al., 2019).

Total β -glucan content of shiitake and all generated extracts was determined using a commonly used enzymatic method (Gil-Ramirez & Soler-Rivas, 2014). The initial raw powder contained 29% (w/w) β -glucans representing the 69% of the total carbohydrates quantified being chitins in lower concentrations (5.2%). Both values were in concordance with several works (Roncero-Ramos, Mendiola-Lanao, Perez-Clavijo, & Delgado-Andrade, 2017; Morales et al., 2018a; Morales et al., 2019) although they were slightly higher than others (Nitschke et al., 2011; Sari, Prange, Lelley, & Hambitzer, 2017). HWE extracted lower β -glucan amounts than UAE or SWE except for the SWEs carried out during 60 min (Figure 2a). In fact, subcritical water extractions longer than 15 min were detrimental to get β -glucan-enriched fractions, opposite to results obtained after UAE extractions. In the latter case, 60 min extraction yielded fractions with β -glucan concentrations similar to those obtained with only 15 min SWEs. Moreover, HWE extracted from 6 to 9% of the chitins present in shiitake mushrooms and similarly UAE extracted 5 to 15%. Only SWE at 15 min showed the highest recovery (35%). However, since chitins are completely insoluble in water, high recoveries were not expected. The extracted compounds might be derivative products from the chitin hydrolysis or depolymerization induced by high temperatures, pressures and/or ultrasounds as suggested in previous works (Palanisamy et al., 2014; Morales et al., 2018a; Morales et al., 2019). SWEs displayed a significant decrease in chitin yield (Figure 2b) similar to those recorded for β -glucans. It might indicate that extraction time was excessive and therefore the

filters from the extraction cell were blocked and no proper extraction was made. However, it might also suggest that the excessive time at 200°C induced hydrolysis and degradation of these two kinds of polysaccharides into lower molecular weight products that were not precipitated as polysaccharides in the analyzed extracts.

In order to test whether the different utilized extraction methodologies showed certain structure selectivity, the β -glucan contents were also evaluated using two additional methods developed to detect (1 $\rightarrow$ 3)- or (1 $\rightarrow$ 3),(1 $\rightarrow$ 6)- β -glucans (Ko & Lin, 2004; Nitschke et al., 2011). The amounts of extracted β -glucans were slightly different depending on the methodology utilized *e.g.* after 15 min, HWE and SWE yielded fractions with similar (1 $\rightarrow$ 3)- β -glucan concentrations (Figure 3a), however, in the SWE fraction 84% of them were pointed as (1 $\rightarrow$ 3),(1 $\rightarrow$ 6)- β -glucans while the HWE fraction apparently contained only 21% of β -glucans with (1 $\rightarrow$ 6) (Figure 3b). Similarly, after 60 min extraction, UAE fractions showed lower (1 $\rightarrow$ 3)- β -glucans than HWE fractions but most of them were detected as (1 $\rightarrow$ 3),(1 $\rightarrow$ 6)- β -glucans, reaching levels similar to SWE fractions extracted for 15 min. These results suggested that HWE was extracting more linear glucans than SWE or UAE as linear β -glucans are usually bound by (1 $\rightarrow$ 3)- β -linkages while SWE or UAE extracted more branched (1 $\rightarrow$ 3),(1 $\rightarrow$ 6)- β -glucans. However, branched glucans are more soluble and therefore they are easily extractable while the linear (1 $\rightarrow$ 3)- β -glucans remain firmly attached to the fungal cell wall, requiring stronger extraction methods. For this reason, SWE or UAE were expected to extract more linear glucans than milder treatments (HWE). An explanation for this could be that the (1 $\rightarrow$ 3)- β -glucans obtained with HWE presented lower molecular weight being consequently more soluble than those glucans extracted with SWE or UAE (as noticed with HPSEC, see later). Moreover, a quantitative mismatch was also noticed between the values obtained with the enzymatic method calculating the total β -glucan content and the fluorimetric/colorimetric determinations. For instance, SWE (15 min) fractions contained 27.1 g/100g total β -glucans (Figure 2) but only 9% of them exhibited fluorescence indicating the presence of β -(1 $\rightarrow$ 3) linkage (Figure 3a) and 7% of them were detected by the colorimetric method as (1 $\rightarrow$ 3), (1 $\rightarrow$ 6)- β -glucans (Figure 3b). But, according to previous publications most of the β -glucans present in shiitake mushrooms showed β -(1 $\rightarrow$ 3),(1 $\rightarrow$ 6)

structures such as for instance lentinan (Nitschke et al., 2011) thus, these determined percentages were too low for a main component. Moreover, the SWE fraction obtained after 15 min contained only 15.5 g/100 g polysaccharides (Table 1) and 2.5 g of them were pointed as chitins (Figure 2b). These observations might indicated that selected extraction technologies were extracting more polysaccharides than noticed or that the enzymatic determination might overestimate the total β -glucan concentration. Other reports also noticed significantly lower β -glucans values in *L. edodes* when fluorimetric methods were utilized compared to the enzymatic procedure (Gründemann et al., 2015). Therefore, the use of indirect colorimetric/fluorimetric determinations should never be considered (in complex mixtures as these extracts) as a precise determination method since their sensitivity might be influenced by the presence of many interference compounds as suggested for the fluorimetric determination (Gil-Ramirez et al., 2019). Thus, in order to define the fraction compositions more precise analytical tools were utilized.

3.2. Combining extraction methodologies

Individual extractions indicated that SWE carried out during 15 min and UAE during 60 min were adequate to extract more β -glucans than either HWE or SPE methods. However, shiitake contained approx. 34.3% polysaccharides and they were not all extracted. Thus, combination of both technologies UAE+SWE were tested to improve the β -glucan content of obtained fractions. Moreover, a pretreatment of the shiitake raw material with supercritical CO₂ was also tested because on the one hand, CO₂ generates an acid environment that might enhance cell structures breakdown and on the other hand, it extract lipid compounds that are within the mushroom dry matter (chitins and β -glucans). The latter consequence combined with the high pressure used during SFE might alter cell wall structure facilitating its disruption and the extraction of structural polysaccharides. Therefore, pretreated (SFE) material was submitted to UAE or SWE selecting the optimal extraction times.

The three tested combinations of extracting methods showed very high yields (Table 1), almost doubling those obtained with individual extractions, particularly UAE+SWE. However, not all the

extracted material precipitated afterwards and they were not all polysaccharides. Combination of UAE+SWE showed results similar to SFE+SWE, apparently both pre-treatments facilitates the subsequent SWE extracting approx. 31% more polysaccharides than only SWE. SFE pre-treatment also improved approx. 41% the polysaccharide levels obtained in UAE fractions although they still contained less than UAE+SWE or SFE+SWE fractions. The combinations were more effective than SPE methods (5.1%) or other extraction technologies used to obtain polysaccharides-enriched fractions such as MAE (10.5%) (Gil-Ramirez et al., 2019).

Apparently, UAE+SWE or SFE+SWE extracted almost all the carbohydrates present in the mushroom (Table 2) and according to the enzymatic protocol they also managed to extract all the β -glucans yielding fractions containing approx. 35% β -glucans. However, the results from the fluorimetric method indicated that shiitake contained 10.4% (1 $\rightarrow$ 3)- β -glucans and the combination of methodologies extracted slightly less compounds yielding fractions with up to 8% (1 $\rightarrow$ 3)- β -glucans. The combination SFE+UAE was in this case more adequate to concentrate this type of compounds on fractions including up to 9% (1 $\rightarrow$ 3)- β -glucans where one third of them were (1 $\rightarrow$ 3),(1 $\rightarrow$ 6)- β -glucans. In fact, the three combinations extracted similar concentrations of (1 $\rightarrow$ 3),(1 $\rightarrow$ 6)- β -glucans but they all generated fractions that contained more β -glucans than individual extractions. The differences between the selected combinations were more pronounced in their capacity to extract chitin-derivatives compounds. UAE+SWE extracted almost 83% of the chitins detected in the mushroom yielding fractions with more than 5% chitin-derivatives. SFE+SWE were less effective and significantly different than SFE+UAE that extracted even less derivatives. Comparing to other technologies, chitins were present in MAE extracts in approx. 2% (Smiderle et al., 2017) therefore, the pretreatment with SFE or UAE particularly before SWE seemed to be a more interesting protocol to extract β -glucans and soluble chitins-derivatives than application of individual extraction methodologies, probably because they both facilitate the disruption of the material for a subsequent subcritical water extraction.

However, similar quantitative discrepancies between the enzymatic and the fluorimetric determinations were detected as noticed within the fractions obtained with individual extractions.

Thus, the enzymatic method was carried out using 100% starch and schyzophyllan as respectively (1→4),(1→6)- α and (1→3),(1→6)- β -glucan standards and mixed as 50% starch:sea sand, 50% starch:schyzophyllan and 50% schyzophyllan:sea sand (w/w). Results indicated that, when only β -glucans were present in the mixtures, quantitative determinations were accurate (only 7% error) but, the amount of α -glucans was underestimated particularly when mixed with schizophyllan inducing a β -glucan overestimation (approx. 14.2%) since the amount of β -glucans is calculated by subtracting the α -glucan levels to the total glucan concentration. Therefore, the enzymatic, fluorimetric or colorimetric methods should be only used for preliminary assessment and more precise determinations were carried out in order to identify the compounds present in the obtained fractions.

3.3. Chemical composition of the obtained fractions

The monosaccharide composition of the polysaccharide-enriched extracts obtained with individual and combined extraction technologies was analyzed by GC-MS. Results pointed out the presence of mannose, galactose, and glucose, being the latter in high levels (Table 3). Higher extraction periods led to higher amounts of glucose in HWE extracts, while the glucose content of UAE extracts (and of the other two monosaccharides) was almost independent of the extraction time. The glucose levels were in concordance with β -glucan determinations, *e.g.* SWE extracted higher β -glucan amounts at 15 min than at longer extraction times as well as higher glucose levels. UAE+SWE extracts showed higher glucose levels than UAE but similar to SWE extracts suggesting that probably with the first extraction (UAE) only part of the glucans were extracted and in the second extraction an approximately 20 % more was further extracted to yield the same values as with SWE alone. Heteropolymers including mannose and galactose in their structure are usually easily water-soluble since they are weakly attached to other molecules from the fungal matrix (Smiderle et al., 2008). HWE extracts obtained with short extraction times showed higher levels of mannose than longer extractions. On the contrary, SWE extracts (15 min) contained lower mannose and lower galactose contents, being glucose more concentrated suggesting that SWE might show certain specificity to isolate β -glucans than other heteropolymers. UAE extracts showed higher

levels of mannose and galactose and lower glucose than SWE (15 min), suggesting a more heterogeneous composition than the SWE extract. The use of SFE pretreatment slightly enhanced the β -glucan selectivity noticed for SWE since SFE+SWE extracts showed a slightly higher percentage of glucose and lower galactose levels. This combination was more specific than UAE+SWE where more mannose and less glucose was noticed.

The different monosaccharide composition noticed depending on the extraction methodology utilized suggested the presence of different polymers, therefore, the extracts were also analyzed by HPSEC. Results showed heterogeneous elution profiles (Supplementary figure S1) confirming the presence of polysaccharides with different molecular mass. Similar profiles were observed for HWE extracts obtained at different extraction times (Fig. S1a). UAE extracts obtained after 60 min extractions were also slightly different than the other extracts obtained at shorter extraction times (Fig S1b) but differences were more pronounced for SWE extracts. Those fractions obtained after 15 min extraction showed a peak with maximum at approx. 57 min indicating the presence of medium molecular weight compounds (Fig. S1c). With increasing extraction times, their profile shifted to other peaks with maxima approx. at 60 min indicating the presence of lower molecular weight compounds and therefore, suggesting degradation provoked by the large extraction method. UAE+SWE and SFE+SWE extracts showed a profile similar to SWE extracts while in SFE+UAE extracts seemed to increase the amount of high molecular weight compounds (R.T. approx. 45 min) compared to UAE extracts. The high molecular weight compounds noticed in UAE-related extracts were lacking in SWE extracts suggesting that the polymers extracted with SWE showed a more narrow size distribution (medium molecular weight) while UAE-related extracts contained a more heterogeneous size distribution.

Moreover, to identify the compounds present in polysaccharide-enriched extracts from the individual and combined extraction methodologies, NMR studies were carried out and compared with literature data (Supplementary figure S2). The extracts contained a mixture of polysaccharides, including α - and β -glucans and a heteropolymer composed of mannose and galactose (Abreu et al., 2019; de Jesus et al., 2018; Smiderle et al., 2008). Although samples were precipitated with cold

ethanol, trehalose signals were observed (δ 92.9/4.82 ppm and 92.6/4.81 ppm). Trehalose is a disaccharide, also known as mycose, and that is commonly found in large amounts in mushrooms. This sugar was also detected in *Pleurotus ostreatus* extracts obtained by SWE in previous works (Smiderle et al., 2017) and could be removed from the polysaccharide fractions after performing dialysis (data not shown).

Intense signals relative to C-1 of α -D-Glcp (δ 99.3-99.5/4.97-4.98) and to C-3 *O*-substituted (δ 82.8-82.9/3.54-3.55) were observed, suggesting the presence of (1 $\rightarrow$ 3)- α -glucan structures, that were also previously reported and isolated from other mushrooms such as *Fomitopsis betulina* (de Jesus et al., 2018). Moreover, the extracts showed intense ^{13}C signals arising at 102.4-103.7 ppm and ^1H signals at 4.14-4.44 ppm confirming the presence of C-1 of glucans in β -configuration (Liu et al., 2014), as well as signals at the range of δ 85.8-87.0 (^{13}C) and at δ 3.23-3.39 (^1H) related to C-3 *O*-substituted. In addition, inverted signals at δ 68.1-68.5/3.89-3.99 and δ 68.1-68.5/3.37-3.50 confirmed CH_2 *O*-substitution of the same units, being in the concordance with the scientific literature, where the mainly studied glucan from shiitake mushrooms is a branched (1 $\rightarrow$ 3)(1 $\rightarrow$ 6)- β -glucan.

Small intensity signals of α -D-Galp were observed in almost all spectra at 98.4-98.6/4.63-4.65 ppm as well as signals of β -D-Manp at δ 101.1-101.5/4.82-4.84 ppm, being in concordance with the results of monosaccharide composition and suggesting the existence of a heteropolymer consisting of galactose and mannose.

According to these results, it was possible to observe that the polysaccharide-enriched fractions obtained from different extraction methods presented similar polysaccharide composition: (1 $\rightarrow$ 3)- α -glucans, (1 $\rightarrow$ 3)(1 $\rightarrow$ 6)- β -glucans, and a heteropolymer composed of mannose and galactose. The main difference among them seemed to be the proportion of each polysaccharide extracted.

3.4. Biological activities of the obtained extracts

The production of β -glucan-enriched fractions should be encouraged for the production of functional foods because fungal β -glucans are biologically active molecules with many interesting

biological activities. However, their beneficial properties can be influenced by their extraction procedure since some of them might modify the tridimensional structure of the polymer (Benito-Roman et al., 2016b). Therefore, the hypocholesterolemic and immunomodulatory activities of the precipitated extracts with the highest β -glucan contents were selected to evaluate whether after the extraction treatments, they still maintain their biological activities.

Water soluble extracts from several mushrooms including soluble β -glucans were able to inhibit the key enzyme of the cholesterol metabolism (Gil-Ramirez et al., 2017), therefore, obtained extracts were tested as HMGCR (3-hydroxy-3-methylglutaryl coenzyme A reductase) inhibitors. Results indicated that SFE+UAE and SFE+SWE extracts showed inhibitory capacity similar to 0.5 mg/mL pravastatin, the statin utilized as positive control (Table 4). The UAE+SWE extract exhibited a slightly lower capacity. The combination of these two extraction methodologies seemed to generate extracts with lower inhibitors concentration or to be slightly detrimental for the structure of the HMGCR inhibitors but still, it was better combination than HWE where half of the inhibitory activity was lost. Gil-Ramirez et al. (2013a) studied the HMGCR inhibitory activity of SWE extracts obtained from *L. edodes* and indicated that extracts obtained at 150 °C (5 cycles 5 min) showed less inhibitory activity than similar extracts obtained at 25°C probably because the inhibitors were thermal sensitive. However, SFE+SWE or UAE+SWE extracts still retained very high inhibitory activity. Perhaps, the pre-treatment with UAE or SFE prior to SWE protected/modulated the inhibitors structure making them more active or less susceptible to thermal degradation or perhaps, the *L. edodes* strain was different than the one utilized in the previous work as differences between varieties or cultivation methods seemed to influence their inhibitory properties too (Gil-Ramirez et al., 2013a).

The immunomodulatory activities of the extracts obtained with combined methodologies were also tested as their capacity to reduce the secretion of pro-inflammatory cytokines in macrophages differentiated from THP-1 human monocytes cell line. The preliminary experiments to assess the extract cytotoxicity indicated that when applied up to 200 μ g/mL viability of THP-1 macrophages was not affected (data not shown). Thus, the immunomodulatory activity was tested in two subtoxic

concentrations (100 and 200 µg/mL). The THP-1 macrophages stimulated with LPS (positive control) exhibited a significant release of the three pro-inflammatory cytokines studied (1295 pg/mL TNFα, 4586 pg/mL IL-1B and 1837 pg/mL IL-6) compared to non-stimulated cells (negative control) (Figure 4). Addition of the extracts obtained with combined methodologies significantly reduced the amount of TNFα liberated in the media. When applied at the highest tested concentration a 24 to 32% reduction in TNFα was noticed (down to 883 pg/mL in the case of SFE+UAE), however, the extract obtained after 60 min HWE did not effectively modulate any response. SFE+UAE seemed to be more effective because it induced the mentioned TNFα reduction and was the only extract inducing a 25% reduction (3462 pg/mL) in IL-1β secretion when applied at 200 µg/mL. The IL-6 release was also inhibited approx. 25% by SFE+UAE and SFE+SWE (1420 and 1343 pg/mL, respectively) extracts being the latter also significantly effective when applied at lower concentration. However, SWE extracts obtained from *L. edodes* at 50 °C (5 cycles 5 min) reduced approx. 90% the release of IL-6, IL-1b and 20% TNFα while if the temperature was higher (200 °C) only a slight reduction in IL-6 was noticed (Gil-Ramirez et al., 2013b). Thus, the temperature utilized in the production of the SFE+SWE extract could have impaired their beneficial immunomodulatory properties. Thus, SFE+UAE was a more adequate combination of methodologies to maintain the immunomodulatory properties since its extract could reduce the liberation of all pro-inflammatory cytokines tested but, their activity might be influenced by the temperature utilized.

4. Conclusion

In conclusion, submission of shiitake mushrooms to UAE or SFE followed by UAE or SWE were more effective methods to obtain β-glucan-enriched fractions than individual UAE or SWE extractions. The generated fractions contained approx. 20% polysaccharides although according to enzymatic determinations they included approx. 34% β-glucans. Fluorimetric/colorimetric methods pointed out lower amounts of (1→3) and (1→3),(1→6) linked β-glucans. These discrepancies might be caused because they were determined by indirect rough methods that should be considered as

preliminary estimations. More accurate analytical methods indicated that UAE fractions contained more heterogenic composition (more mannose and galactose and less glucose contents) and polymers of higher molecular weight than SWE (15 min) or SFE+SWE fractions. UEA-related extracts (*e.g.* 60 min or SFE+UAE, etc.) also extracted lower chitin-derivative contents than SWE-related extracts (15 min, UAE+SWE, SFE+SWE, etc). However, the main differences among the fractions seemed to be the extracted polysaccharides ratio since they all contained (1→3)- α -glucans, (1→3),(1→6)- β -glucans, and heteropolymers composed of mannose and galactose. Moreover, the obtained fractions might be of interest to design functional foods with hypocholesterolemic properties since they showed high HMGCR inhibitory activity, even higher than other reported extracts obtained at high temperatures similar to those used in SFE+SWE or SFE+UAE (Gil-Ramirez et al., 2013a). But, the immunomodulatory properties of the extracted β -glucans might be compromised because although they were still able to reduce secretion of TNF α , IL-1 β and IL-6 in macrophage cell lines, only the SFE+UAE fractions managed to reduce them approx. 20% while other extracts obtained using SWE at lower temperatures were reported more effective (Gil-Ramirez et al., 2013b).

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Declaration of interest

None.

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Table captions

Table 1. Extraction yields, polysaccharides and other carbohydrates contents obtained in the fractions extracted from shiitake using individual and combined extraction technologies. Different letters (a-g) denote significant differences ($P < 0.05$). *Calculated by subtracting total polysaccharide levels to the total carbohydrate values from the extracted fractions obtained before polysaccharide precipitation

Table 2. Carbohydrates determined in shiitake powder and the fractions obtained by SPE and combined technologies (UAE+SWE, SFE+UAE and SFE+SWE). Different letters (a-d) denote significant differences ($P < 0.05$) between values of the same column.

Table 3. Monosaccharide composition (%) of the precipitated extracts obtained from shiitake by the different individual and combined technologies.

Table 4. HMGCR inhibitory activity (%) of the obtained precipitated extracts obtained by HWE during 60 min (HWE60') and combination of advanced methodologies. Different letters (a-c) denote significant differences ($P < 0.05$) between samples.

Figure captions

Figure 1. Extraction workflow followed to obtain different fractions and extracts from shiitake powder. Continuous line indicate single extractions and dotted lines indicate the order followed when two technologies were combined. (SPE, steam pressurized extraction; SFE, supercritical fluid extraction; UAE, ultrasound assisted-extraction; SWE, subcritical water extraction).

Figure 2. Total β -glucan (a) and chitin (b) content in the fractions obtained after HWE, UAE and SWE from shiitake mushrooms. Different letters denote significant statistical differences ($P < 0.05$) between fractions obtained by the same extraction technology at different times (A-D) and obtained by different extraction technologies at the same time (a-c).

Figure 3. Content of (a) (1 $\rightarrow$ 3)- β -glucans and (b) (1 $\rightarrow$ 3),(1 $\rightarrow$ 6)- β -glucans in the fractions obtained after HWE, UAE and SWE from shiitake mushrooms. Different letters denote significant statistical differences ($P < 0.05$) between fractions obtained by the same extraction technology at different times (A-B) and obtained by different extraction technologies at the same time (a-c).

Figure 4. Levels of a) TNF α , b) IL-1 β and c) IL-6 secreted by THP-1/M activated with LPS in presence of HWE (60 min), UAE+SWE, SFE+UAE and SFE+SWE extracts. Positive control: cells stimulated with LPS but in absence of extract. Negative control: non LPS-activated cells. Different letters (a-c) denote significant differences ($P < 0.05$) between samples.

Table 1.

Technology	Extraction time (min)	Yield of extracted matter/ 100 g shiitake (% w/w)	Yield of precipitated matter/ 100 g extracted fraction (% w/w)	Yield of precipitated matter/ 100 g shiitake (% w/w)	Total polysaccharides in extracted fractions (g/100g)	Other carbohydrates in extracted fractions (g/100g)*
Hot water extraction (HWE)	15	37.99±5.35 ^{de}	20.29±2.88 ^c	7.71±1.09 ^g	5.02±0.71 ^c	9.63
	30	34.58±2.22 ^c	19.76±1.20 ^c	6.83±0.41 ^g	4.56±0.27 ^c	18.49
	45	39.96±0.06 ^{de}	21.18±0.30 ^{de}	8.46±0.12 ^g	4.02±0.06 ^c	30.09
	60	31.24±0.90 ^c	16.59±0.52 ^{ef}	5.18±0.16 ^g	4.78±0.15 ^c	41.79
Ultrasound-assisted extraction (UAE)	15	45.74±1.42 ^d	27.26±0.60 ^d	12.47±0.27 ^g	8.59±1.41 ^{bc}	13.74
	30	56.19±2.81 ^c	30.90±0.76 ^{cd}	17.36±0.43 ^c	9.37±2.92 ^{bc}	21.04
	45	58.52±2.22 ^c	30.92±1.95 ^{cd}	18.09±1.14 ^c	7.76±1.31 ^c	29.73
	60	60.36±1.48 ^c	34.56±3.17 ^c	20.86±1.91 ^c	10.14±2.46 ^{bc}	36.50
Subcritical water extraction (SWE)	15	73.21±0.91 ^{ab}	52.88±2.12 ^b	38.71±1.55 ^c	15.54±2.18 ^{ab}	12.85
	30	75.31±0.91 ^{ab}	23.16±0.56 ^{de}	17.44±0.42 ^c	6.80±0.94 ^c	31.64
	45	70.58±3.82 ^b	26.96±0.99 ^{de}	19.03±0.70 ^c	7.93±1.07 ^c	32.51
	60	64.49±2.62 ^{bc}	20.05±1.32 ^c	12.93±0.85 ^f	5.92±0.67 ^c	27.79
Steam pressurized extraction (SPE)	20	37.96±0.16 ^{de}	11.42±0.01 ^f	4.34±0.01 ^g	5.13±0.01 ^c	10.16
UAE+SWE	60 + 15	81.20±1.06 ^a	68.88±2.33 ^a	55.93±1.89 ^a	21.14±1.96 ^a	37.97
SFE+UAE	180 + 60	59.73±0.44 ^c	49.42±2.66 ^b	29.52±1.59 ^d	14.46±1.97 ^b	34.46
SFE+SWE	180 + 15	74.95±1.26 ^{ab}	63.54±1.75 ^a	47.62±1.31 ^b	19.88±1.23 ^{ab}	39.27

*Calculated by subtracting total polysaccharide levels to the total carbohydrate values from the extracted fractions obtained before polysaccharide precipitation

Table 2.

	Total carbohydrates		Total β -glucans		(1 $\rightarrow$ 3)- β -glucans		(1 $\rightarrow$ 3),(1 $\rightarrow$ 6)- β -glucans		Chitins	
Sample	g / 100 g fraction	g / 100 g shiitake	g / 100 g fraction	g / 100 g shiitake	g / 100 g fraction	g / 100 g shiitake	g / 100 g fraction	g / 100 g shiitake	g / 100 g fraction	g / 100 g shiitake
Shiitake powder	-	42.10 $\pm$ 1.17 ^a	-	28.85 $\pm$ 0.43 ^a	-	10.40 $\pm$ 0.22 ^a	-	3.22 $\pm$ 0.01 ^a	-	5.19 $\pm$ 0.13 ^a
SPEmethod	15.29 $\pm$ 3.33 ^b	5.80 $\pm$ 1.26 ^c	6.32 $\pm$ 0.29 ^b	2.40 $\pm$ 0.11 ^d	2.94 $\pm$ 0.01 ^b	1.12 $\pm$ 0.01 ^c	0.50 $\pm$ 0.01 ^b	0.19 $\pm$ 0.01 ^c	1.07 $\pm$ 0.09 ^d	0.41 $\pm$ 0.03 ^e
UAE+SWE	59.11 $\pm$ 1.22 ^a	47.99 $\pm$ 0.99 ^a	35.97 $\pm$ 0.43 ^a	29.21 $\pm$ 0.35 ^a	8.33 $\pm$ 0.18 ^a	6.76 $\pm$ 0.15 ^b	2.50 $\pm$ 0.32 ^a	2.03 $\pm$ 0.26 ^b	5.30 $\pm$ 0.18 ^a	4.30 $\pm$ 0.15 ^b
SFE+UAE	48.92 $\pm$ 3.43 ^a	29.22 $\pm$ 2.05 ^b	32.17 $\pm$ 0.28 ^a	19.22 $\pm$ 0.17 ^c	9.09 $\pm$ 0.94 ^a	5.43 $\pm$ 0.56 ^b	3.03 $\pm$ 0.24 ^a	1.81 $\pm$ 0.14 ^b	3.86 $\pm$ 0.05 ^c	2.31 $\pm$ 0.03 ^d
SFE+SWE	59.15 $\pm$ 5.23 ^a	44.33 $\pm$ 3.91 ^a	34.82 $\pm$ 0.32 ^a	26.10 $\pm$ 0.24 ^b	8.12 $\pm$ 0.66 ^a	6.09 $\pm$ 0.49 ^b	2.51 $\pm$ 0.14 ^a	1.88 $\pm$ 0.10 ^b	4.76 $\pm$ 0.14 ^b	3.57 $\pm$ 0.10 ^c

Table 3.

Technology	Extraction time (min)	Monosaccharides (%)		
		Mannose	Glucose	Galactose
Hot water extraction (HWE)	15	21.9	68.6	9.5
	30	16.1	71.2	12.7
	45	9.3	81.5	9.2
	60	9.2	81.3	9.5
Ultrasound-assisted extraction (UAE)	15	22.3	62.0	15.7
	30	22.2	61.8	16.0
	45	22.8	62.9	14.3
	60	24.3	63.1	12.6
Subcritical water extraction (SWE)	15	10.4	83.9	5.7
	30	12.2	82.1	5.7
	45	25.0	68.5	6.5
	60	26.5	63.9	9.6
Steam Pressurized extraction (SPE)	20	13.6	74.0	12.4
UAE+SWE	60+15	12.2	82.8	5.0
SFE+UAE	180+60	11.4	81.4	7.2
SFE+SWE	180+15	10.0	85.3	4.7

Table 4.

Sample	HMGCR inhibition (%)
HWE60'	42.52±0.01 ^c
UAE+SWE	84.41±6.98 ^b
SFE+UAE	89.03±0.48 ^{ab}
SFE+SWE	87.32±1.89 ^{ab}
Pravastatin	99.21±0.36 ^a

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Supplementary material:

Figure S1. HPSEC elution profiles of a) HWE, b) UAE and c) SWE fractions obtained at different extraction times (15 to 60 min) and d) SPE and combined fractions.

Figure S2. HSQC-DEPT35 NMR spectra of extracts obtained after a) HWE 60 min, b) UAE 60 min, c) SWE 15 min, d) UAE+SWE, e) SFE+UAE and f) SFE+SWE. Experiments were performed in DMSO at 70 °C (chemical shifts are expressed in δ ppm).

Supplementary figure 1 (S1).

Supplementary figure 2 (S2).