Extraction of bioactive compounds against cardiovascular diseases from *Lentinula edodes* using a sequential extraction method

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Abstract

Three extraction methods were sequentially combined to obtain fractions from *Lentinula edodes* (shiitake mushrooms) containing bioactive compounds against cardiovascular diseases (CVDs). Fruiting bodies were first extracted with plain water, obtained residue was then submitted to supercritical fluid extraction (SFE) and remaining residue submitted to hot water extraction. Sequential design allowed re-utilization of the non-extracted material as raw material for the successive extractions increasing extraction yields and separating interesting compounds. Obtained fractions contained different amounts of ß-glucans, chitins, eritadenine, lenthionine, ergosterol, proteins/peptides and phenolic compounds conferring them different bioactivities. Water soluble fractions showed high antioxidant activities ($ABTS^{+\bullet}$ and $DPPH^{\bullet}$ scavenging capacity and reducing power), they were also able to inhibit one of the main enzymes involved in hypertension (angiotensin-I converting enzyme) and the key enzyme of cholesterol metabolism (3-hydroxy-3-methylglutaryl coenzyme A reductase). The latter inhibitory activity was also noticed in SFE extracts although ergosterol and other lipid-like molecules were isolated. Dietary fibers were separated in the third extraction. Therefore, with this sequential extraction procedure bioactive compounds against CVDs can be selectively separated from a single batch of shiitake powder.

Keywords: Supercritical CO_2 extraction; Shiitake mushrooms; Cholesterol; Antioxidant activity, HMGCR inhibition**.**

Introduction

Cardiovascular diseases (CVDs) are still one of the leading causes of mortality in developed countries. CVDs are associated with multiple factors such as high triglycerides and low density lipoprotein cholesterol (LDLc) levels, LDL oxidation, increased platelet aggregation, hypertension and smoking.[1,2]

Nowadays, functional foods containing phytosterols or β-glucans are being commercialized to reduce low to moderate hypercholesterolemia because these compounds showed the ability of impairing exogenous cholesterol absorption. Hypotensive foods are also marketed containing specific peptides able to inhibit the angiotensin I converting enzyme (ACE)[3] and many juices and functional drinks indicate that they contain many compounds with high antioxidant properties. These compounds might inhibit LDL oxidation, prevent atheroma plaque formation etc. contributing to reduce the risk of CVDs. However, a more effective novel food may be designed if all compounds are combined and, besides those inhibiting cholesterol absorption, other compounds are incorporated such as those reducing the risk of CVDs by different mechanisms of action.[4]

Edible mushrooms are natural sources of hypocholesterolemic compounds, such as fungal sterols (ergosterol and derivatives) and polysaccharides (β-glucans, chitins, etc.) that according to *in vitro* experiments might inhibit cholesterol absorption and biosynthesis.[4-7] Eritadenine (2(*R*),3(*R*)-dihydroxy-4-(9-adenyl) butanoic acid) from *Lentinula edodes* (shiitake mushrooms) was also able to lower cholesterol levels by acting as an inhibitor of the S-adenosyl-L-homocysteine hydrolase involved in the hepatic phospholipid metabolism.[8,9] When lard was supplemented with extracts containing these compounds and fed to mice, they succeeded to avoid the increasing of cholesterol levels noticed in control mice fed only with lard.[10]

Moreover, water extracts obtained from edible mushrooms such as *Hypsizygus marmoreus* and *Lactarius camphorates* were also able to inhibit ACE because of their hypotensive peptides[11,12] and lenthionine (1,2,3,5,6-pentahiepane), an organosulfur compound responsible for the characteristic flavor of shiitake mushrooms, inhibited platelet aggregation[13]. Water and hot water extracts from other mushroom species also showed interesting antioxidant activities that correlated to their levels of ergothioneine and phenolic compounds.[14-16]

Several methods to isolate or extract each type of those previously mentioned compounds have been already reported. Different β-glucans types are usually isolated by alkali/acid treatments, hot water (120 ^{o}C, 20 min), microwave, pressurized solvent or ultrasound assisted extractions,[17-19] chitins needed more drastic treatments[20] and sterols were isolated by using mixtures of organic solvents or supercritical CO_2 extractions.[21] However, all these methods were optimized to use mushrooms as raw material and for each extraction, a new batch of powdered mushrooms should be used. In the present work, a sequential extraction method was designed to extract from the same batch of mushroom powder a few fractions enriched in different bioactive compounds against CVDs. The residue remaining after one extraction was used to extract other compounds at the following extraction step instead of using different batches of mushroom powder for each type of compound.

Materials and Methods

Biological material

Dry *Lentinula edodes* S. (Berkeley) mushrooms were obtained in a local market (Madrid, Spain). Fruiting bodies were ground as a whole or divided into caps and stipes until a fine powder was obtained and stored at -20 ^{o}C as indicated by Ramirez-Anguiano *et al*., 2007[16] (they were used to analyze separated tissues). Larger amounts of powdered shiitake

77 mushrooms were also purchased from Glucanfeed S.L. (La Rioja, Spain). Obtained powder
78 showed a particle size lower than 0.5 mm, and moisture content lower than 5% and it was
79 stored in darkness at -20 ^{o}C until further use.

80 ***Reagents***

81 Solvents as hexane (95%), chloroform (HPLC grade), methanol (HLPC grade),
82 acetonitrile (HPLC grade) were obtained from LAB-SCAN (Gliwice, Polland) and absolute
83 ethanol, sodium carbonate (Na_2CO_3) and sulfuric acid (H_2SO_4) from Panreac (Barcelona,
84 Spain). Potassium hydroxide (KOH), ascorbic acid, 2,6-Di-tert-butyl-*p*-cresol (BHT), bovine
85 serum albumin (BSA), acetylacetone, p-dimethylaminebenzaldehyde, Trizma base, HCl
86 (37%), trifluoroacetic acid (99%), phenol, Folin Ciocalteu's phenol reagent, 2,2'-azino-bis(3-
87 ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulfate, Angiotensin-I
88 Converting Enzyme (ACE) (5 UN/mL), zinc chloride ($ZnCl_2$) solution (0.1 M) as well as
89 hexadecane, ergosterol (95%), D-glucose, D-glucosamine hydrochloride, gallic acid, 2,2-
90 diphenyl-1-picrylhydrazyl (DPPH), sodium phosphate dibasis dihydrate ($NaH_2PO_4 \cdot 2H_2O$),
91 sodium phosphate monobasic sodium phosphate dibasic anhydre (Na_2HPO_4), potassium
92 ferrycianide ($K_3[Fe(CN)_6]$), ferric chloride ($FeCl_3$) and Trolox were purchased from Sigma-
93 Aldrich Quimica (Madrid, Spain). CO_2 was supplied by Air-Liquid, S.A. (Madrid, Spain). D-
94 Eritadenine (90%) was acquired from Sy Synchem UG & Co. KG (Felsberg, Germany),
95 lenthionine (80%) from Cymit (Barcelona, Spain) and N-(2-aminobenzoyl)glycil-4-nitro-l-
96 phenylanalyl-l-proline (Abz-Gly-Phe(NO_2)-Pro) from Bachem Feinchemikalien (Bubendorf,
97 Switzerland). All other reagents and solvents were used of analytical grade.

98 ***Sequential extractions***

99 A method to obtain different bioactive fractions from powdered shiitake mushrooms
100 was optimized based on three successive extractions (Figure 1). Firstly (Step A), mushroom

101 powder was mixed with water (50 g/L) at room temperature (RT) and vigorously stirred
102 during 1 minute. Afterwards, the mixture was centrifuged (7 min, 7000 rpm, 10ºC) in a
103 Heraus Multifuge 3SR+ centrifuge (Thermo Fisher Scientific, Madrid, Spain). Obtained
104 supernatant (considered extract ExA) was separated from the residue (ReA) and both
105 fractions were freeze dried in a LyoBeta 15 lyophilizer (Telstar, Madrid, Spain).

106 Secondly (Step B), freeze-dried ReA (253 g) was ground, sieved until particles size
107 < 0.5 mm and submitted to supercritical fluid extraction (SFE). ReA was mixed with 1.9 kg
108 of 5 mm diameter stainless steel spheres (a ratio 1:1 (v/v) extract:spheres) in a 2L extraction
109 cell connected to a SFE pilot-scale plant (model SF2000, TharTechnology, Pittsburgh, PA,
110 USA). Pressurized CO_2 was forced to reach supercritical state and injected in the loaded
111 extraction cell. Extracted material was collected in two different separators (S1 and S2) each
112 of 0.5 L capacity with independent control of temperature and pressure. The extraction vessel
113 had a ratio of 5.5 height/diameter (a detailed explanation of the experimental device can be
114 found at Garcia-Risco *et al.*, 2011[22]). Extraction was carried out at 35 MPa and 40 ºC.
115 Pressure of S1 and S2 was maintained at 10 and 6 MPa respectively and temperature in both
116 of them was 40 ºC. The CO_2 flow was set at 3.6 kg/h and during the total extraction time (3
117 h) and it was recirculated. Extracted compounds were precipitated in both separators and at
118 the end of the extraction process, the fractions were dragged with ethanol and immediately
119 submitted to concentration until dryness on a rotary vacuum evaporator. Dried extract (ExB)
120 was stored at -20ºC until further analysis and non-extracted residual material (ReB) was
121 separated from steel spheres by sieving.

122 Finally (Step C), ReB (100 g/L) was submitted to hot water extraction (98 ºC) in a
123 0.5 L flask during 1 hour under vigorous stirring and reflux using a glass steam condenser.
124 The soluble (ExC) and non-soluble (ReC) fractions were separated by centrifugation (7 min,
125 7000 rpm, 10 ºC) and freeze dried.

Carbohydrates determinations

The total carbohydrate content of shiitake mushroom samples or obtained extracts was determined by the phenol-sulphuric acid method adapted from Dubois *et al*. (1956).[23] Briefly, samples (1 mg) were mixed with 1 mL of MilliQ water and stirred during 2 min. The mixtures (25 µL) were added to a 96-well plate, plus 25 µL of 5% phenol solution (w/v) and 125 µL of concentrated H_2SO_4. Afterwards, the plate was sealed and incubated in a water bath at 80 ºC for 30 min. Samples absorbance was read using a M200 Plate Reader (Tecan, Mannedorf, Switzerland) at 595 nm. A standard curve of D-glucose (0.032 to 0.8 mg/mL) was used for quantification.

Chitin content was determined according to Smiderle *et al.* (2017).[18] Firstly, samples were hydrolyzed with 6 M HCl at 100 ºC for 2 h and adjusted to pH 10.0 after cooling down. Then, hydrolyzed samples (250 µL) were treated as described by Rementeria *et al.* (1991).[24] Samples absorbance was measured at 530 nm using an Evolution 600 UV-vis (Thermo Fisher Scientific, Spain) spectrophotometer. A glucosamine hydrochloride standard curve was used for quantification.

The β-glucan content of the obtained mushroom samples and extracts (50 mg) was evaluated by a β-glucan determination kit specific for mushrooms and yeasts (Megazyme®, Biocom, Barcelona, Spain) following the instructions of the user´s manual.

Total phenol content and proteins or peptides determination

The total phenol concentration of samples (10 mg) was determined by the Folin-Ciocalteu method according to the procedure of Ramirez-Anguiano *et al.* (2007).[16] Gallic acid was used as standard for quantification.

148 The total protein concentration of the samples (10 mg/mL) was determined using the
149 Bradford method reagents (Sigma-Aldrich, Madrid, Spain) according to the Instruction
150 Manual. To determine the amount of peptides, extracts were solubilized in water (100
151 mg/mL) and submitted to centrifugation (14000 rpm, 30 min) using Amicon Ultra filter
152 devices with Ultracel 3K membrane (Millipore, Billerica, USA) obtaining a filtrate (< 3 kDa)
153 and a concentrate (> 3 kDa). The latter fraction was submitted to a second centrifugation
154 (14000 rpm, 20 min) using Nanosep centrifugal devices with Omega 10K membrane (Pall
155 Life Sciences, New York, USA), obtaining a filtrate (fraction with MW between 3 and 10
156 kDa) and a concentrate (> 10 kDa). The obtained fractions were freeze dried and mixed with
157 the Bradford reagent as described above. BSA was used as standard (0.0125 to 0.5 mg/mL)
158 for protein quantification.

159 ***Eritadenine and lenthionine determination by HPLC-DAD***

160 Eritadenine was extracted from the samples and analyzed following the procedure of
161 Afrin *et al.* (2016)[25] with modifications. Briefly, samples (1 g) were mixed with 10 mL of
162 60% ethanol (v/v) and stirred for 2 min. The mixture was centrifugated (15 min, 7000 rpm,
163 10 ^{o}C) and the supernatant was collected. Afterwards, 10 mL of 60% ethanol (v/v) was added
164 for a second extraction and both supernatants were pooled together and submitted to vacuum
165 filtration. The filtrate was concentrated on a rotary vacuum extractor at 60 ^{o}C until dryness.
166 Identification and quantification of eritadenine were carried out using a C18 Spherisorb
167 ODS2 4 x 250 mm analytical column with a 5 µm particle size (Waters, Missisagua, Ontario,
168 Canada) coupled to an HPLC system (Pro-Star 330, Varian, Madrid, Spain) with PDA
169 detector (Pro-Star 363 module, Varian, Madrid, Spain). Samples were dissolved in mobile
170 phase (5 mg/mL) and they were injected (10 µL) and developed under a constant flow (0.5
171 mL/min) and an isocratic mobile phase of water:acetonitrile (98:2, v/v 1% TFA). Eritadenine

was quantified at 260 nm using a commercial standard. The compound eluted at 11.6 min and showed the characteristic eritadenine UV-spectrum.

Lenthionine determination was carried out according to the procedure of Hiraide *et al.* (2010)[26] with slight modifications. Basically, samples (50 mg) were mixed with 1 mL of 0.2 M Tris-HCl buffer (pH 8.0) and stirred for 1 h. Afterwards, methanol (0.5 mL) was added, stirred for 2 min and centrifuged (14000 rpm 5 min). The obtained supernatant was collected and the residue submitted to extraction twice. Supernatants were pooled together, diluted with 2.5 mL MilliQ water and filtered using a syringe through a 0.45 µm pore size filter. The filtrate was applied to an ODS cartridge (Waters, Missisagua, Ontario, Canada) preactivated with methanol following manufacturer´s instructions. The cartridge was washed with 1 mL 30% methanol (v/v) and lenthionine was eluted with 1 mL 65% methanol (v/v). Obtained eluate (50 µL) was injected into an HPLC-DAD system (the same column and equipment than above described) and developed using an isocratic mobile phase (65% methanol v/v), a constant flow (0.7 mL/min) and temperature (45 ^{o}C). Lenthionine (retention time 10.4 min) was quantified at 230 nm and identified using a lenthionine commercial standard.

Ergosterol determination by GC-MS-FID

Fungal sterols from samples were evaluated following the procedure described by Gil-Ramirez et al. (2013).[21] The unsaponified fractions obtained (6 mg/mL) were injected into an Agilent HP-5ms capillary column (30 m x 0.25 mm i.d. and 0.25 µm phase thickness). The column was connected to a 7890A System gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), comprising a split/splitless injector, an electronic pressure control, a G4513A autoinjector, a 5975C triple-axis mass spectrometer detector and a GC-MS Solution software. The injector and detector conditions as well as the column temperature program

were those described by Gil-Ramirez *et al.* (2013).[21] Ergosterol was used as standard and hexadecane (10% v/v) as internal standard for quantification.

Antioxidant activities

Mushroom powder and obtained extracts were dissolved in water (0.02 to 0.5 mg/mL) and assayed for their $ABTS^{+\bullet}$ scavenging activity assay. ABTS radical was chemically generated using potassium persulfate and $ABTS^{+\bullet}$ scavenging activity was analyzed spectrophotometrically according to Re *et al*. (1999),[27] measuring changes in absorbance (734 nm) at several concentrations after 15 minutes of incubation at room temperature in darkness. Similarly, samples were also dissolved in methanol (0.02 to 0.5 mg/mL) and mixed with $DPPH^{\bullet}$ (76 µM) to determine their scavenging capacity according to Mau *et al*. (2001) [28]. Absorbance at 517 nm was recorded at several concentrations after 15 min incubation at room temperature in darkness. For both radicals the IC_{50} was established using the linear correlation obtained with increasing sample concentrations and compared with Trolox to express the results as their TEAC values (trolox equivalent antioxidant capacity).

The ferric ion reducing power of the extracts was evaluated according to the method of Oyaizu (1986)[29]. Samples were dissolved in 200 mM sodium phosphate buffer (0.1 to 10 mg/mL) and treated as described by Mau *et al.* (2005)[30]. Absorbance increase was recorded at 700 nm using several concentrations to estimate their EC_{50}. Afterwards, it was expressed as TEAC values to be able to compare with the other antioxidant activities.

HMGCR inhibitory activity

The obtained extracts were solubilized in water, ethanol:water (1:4) or assay buffer (50 mg/mL) and applied (20 µL) 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 utilized as a control for positive inhibition.

ACE inhibitory activity

The Angiotensin-I Converting Enzyme (ACE) inhibitory activity of the obtained extracts was evaluated using the fluorimetric method described by Sentandreu & Toldra (2006)[31] with slight modifications. Basically, 40 µL of different samples dilutions were added to a 96-wells plate, followed by addition of 160 µL of Abz-Gly-Phe(NO_2)-Pro (0.45 mM) and 40 µL of ACE working solution (0.04 U/mL). The plate was incubated during 1 h at 37 ^{o}C measuring the generated fluorescence with excitation and emission wavelengths respectively 355 and 405 nm. Milli Q water was utilized as a control for negative inhibition.

Statistical analysis

Differences were evaluated at a 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, USA). Correlation between different variables was evaluated by computing Pearson correlation coefficient and determination coefficient ($p \leq 0.05$).

Results and discussion

Selection of the starting material

The distribution of compounds with beneficial activities against CVDs within the *Lentinula edodes* fruiting body was investigated as a preliminary study to elucidate whether it was more convenient to use the complete mushroom or perhaps only caps or stipes as raw

material for the extractions. However, when the amount of the main bioactive compounds was determined (Table 1), no significant differences were noticed between tissues. They contained approx. 40 % (w/w) carbohydrates and most of them were β-glucans since chitins were found in lower concentrations. Ergosterol and the other bioactive compounds showed levels similar to previous studies[16,25,32] being lenthionine the compound found in the lowest concentration (0.15 mg/g) but also similar to other publications.[13,26]

The lower part of *L. edodes* stipes (the one is in contact with the cultivation substrate) is considered as a by-product and it is usually discarded during harvesting and not commercialized. However, the stipes showed similar concentrations than the complete fruiting body not only of the bioactive molecules but also of other nutrients such as proteins or carbohydrates. This observation indicated that the wasted stipes could be used as source of bioactive compounds as well as the complete fruiting bodies being an alternative for valorization of these wastes. Nevertheless, since the fruiting bodies were more easily available in larger concentrations the following experiments were carried out using the complete mushrooms.

Sequential extraction yields

The sequential extraction method was designed to optimize the use of shiitake mushrooms or their by-products (as they showed similar composition) as material to extract bioactive compounds. The idea was to consecutively use the residue of one extraction to carry out the next one in order to maximize the number of compounds that could be selectively isolated in each step avoiding the use of larger amounts of mushroom powder for independent extractions.

Water extraction (at room temperature) was selected as first extraction step because some of the bioactive compounds that could be solubilized in this medium were not

thermostable (see later for details). Then, the lipid fraction could be extracted with SFE using moderate temperatures (as second step) and in the third step hot water could be used to extract heat resistant molecules such as dietary fibers improving the intestinal bioavailability (for specific β-glucans) and making them more accessible for colonic microbiota.[33,34]

With the first extraction, water extracted 37 g from 100 g (w/w) of the mushroom powder (Figure 2). This method was previously used to obtain water-soluble polysaccharides[35] and other compounds of lower molecular weight.[36] The lowest yield (1.1 %) was obtained with supercritical CO_2 extraction of the generated residue (1.7% of ReA) since it mostly extracted lipophilic compounds (ExB) and mushrooms have a low lipid content. Moreover, supercritical CO_2 exhibit a high selectivity enabling high recoveries of specific fatty acids, sterols and derivatives from fungal or other matrices.[37] Nevertheless, the obtained yield was similar to those described in previous publications where SFE extractions were carried out directly from fruiting bodies[38,39] indicating that SFE extractions could be also carried out using the residues obtained after the first extraction step. The last step, a hot water extraction (98 ^{o}C), generated a fraction (ExC) containing 20 % (w/w) of ReB (12 % of the initial mushroom powder) leaving still a large insoluble fraction (ReC) encompassing 49.5% of the starting material. This procedure is usually utilized to extract certain polysaccharides from the rest of non-soluble dietary fibres that remain in the residual fraction.[40,41]

Distribution of bioactive compounds within the obtained fractions

The levels of the different fungal polysaccharides were evaluated in all the obtained fractions. Results indicated that the water insoluble fractions contained higher levels of total carbohydrates, β-glucans and chitins than water-soluble or SFE fractions (Figure 3a). The mushroom powder contained 40.7 % (w/w) total carbohydrates where 29.3 % of them were

β-glucans and 6.0 % chitins suggesting that the remaining low percentage (approx. 5 %) should include oligosaccharides, sugars and perhaps small amounts of α-glucans or other heterosaccharides. Total carbohydrates values were in concordance with the literature.[42] β-glucans and chitins were slightly higher than some reports[43,44] but in the same range than others. [45,46,47] Differences might be due to the different methodologies utilized for their determination.

The water extract (ExA) contained a lower content of total carbohydrates and more than half of them were probably sugars and oligomers since they are easily solubilized in water and their β-glucan content was also lower than in the mushroom powder (10.8 %). Although in low amounts, chitins were also detected but they might be degradation products or low molecular weight derivatives from chitins since the latter compounds are completely insoluble in water. These derivatives were also noticed in other reports[17,20] and they might be involved, together with the water soluble β-glucans, in the interesting biological activities noticed in mushroom water extracts. Water soluble β-glucans, α-glucans and fucomannogalactans were pointed as compounds potentially involved in the HMG-CoA reductase inhibitory activity noticed *in vitro* for water soluble extracts.[36] Moreover, chitin oligomers (water-soluble low molecular weight chitin (LMWC) and chitooligosaccharides (COs) derivatives) could be involved in the hypocholesterolemic properties noticed for these extracts.[48]

ReA and ReB showed a very similar carbohydrates profile including approx. 39-40% ß-glucans and 7-8% chitins indicating that the supercritical CO_2 extraction did not significantly influenced the polysaccharide composition of the residue obtained after water extraction and none of them was extracted.

Alkaline/acid or hot water extractions are usually required to achieve fractions with high polysaccharides yields.[35] The hot water extraction did not succeed to separate β-glucans

from chitins. The β-glucans extracted with hot water accounted for approx. less than half of the total carbohydrates noticed in ExC. As probably most of the oligosaccharides and monosaccharides were previously extracted in the ExA, the rest of carbohydrate content could be due to α-glucans or other heteropolysaccharides characteristic from *L. edodes* that were also extracted. Shiitake β-glucans such as lentinan, are usually isolated using hot water extractions although their yields could be enhanced if alkaline media are used. However, alkalis were not recommended since they interfere with the lentinan 3D structure, essential for its biological activities.[49] Hot water soluble polysaccharides were suggested to decrease the oxidative damage related to hypercholesterolemia and modulating immune system.[50-52]

Furthermore, approx. 2/3 of the carbohydrates present in the residue ReC were β-glucans, and chitins (10.2 % w/w) that remained being part of the insoluble fractions. They were not extracted neither with cold/hot water or supercritical CO_2 since their concentrations in all the analyzed residues were almost the same. Their complex-forming capacity and polymeric structure are responsible for their extremely low solubility in many simple solvents.[53] However, this characteristic confers them the ability to act as dietary fibres remaining undigested in the intestine and acting on the colonic flora as prebiotic or as hypocholesterolemic fibers.[48,54]

The total protein content measured in shiitake mushroom was in the range of previously reported studies since they might change from approx. 13 to 23 % depending on the cultivation conditions, developmental stage or commercial strain.[55-57] The highest protein content (Figure 3a) was obtained in ExA, indicating that a considerable amount of Shiitake proteins might be easily extracted with cold water, probably because free proteins, peptides and amino acid derivatives are included in this fraction. Indeed, in this fraction higher amounts of small proteins and peptides (8.57 mg/g) (with molecular weight (MW) between 10 and 3 kDa) than larger proteins (5.88 mg/g) (MW > 10 kDa) were noticed. It also

contained a low amount of peptides and N-containing compounds with MW < 3kDa (0.83 mg/g). But, proteins might be also bound to polysaccharides as glycoproteins or proteoglucans impairing their extraction therefore, other proteins were found in the residues. The high pressure (and/or mild temperature) utilized during SFE extraction seemed to partially denature proteins since ReB protein levels were not the sum of those noticed in the fractions from the following extraction step (Table 2). Nevertheless, the ExC obtained still contained high protein levels and only low amounts remained in ReC (Figure 3a) suggesting that hot water enhanced extraction of many more proteins or different ones from those found in ExA. Nevertheless, the temperature selected might have also influenced proteins structure generating breaking down products from large (5.4 mg/g MW>10 kDa) and smaller proteins particularly those with MW between 10 to 3 kDa since the ExC contained lower content (1.84 mg/g) than ExA and the fraction with lower MW contained more peptides (1.74 mg/g MW <3 kDa).

Eritadenine is a water-soluble alkaloid therefore it was mainly extracted with cold water when compared with the amount noticed in the mushroom powder (Table 2). The minimal amount that remained in the residues was then almost completely extracted with hot water. These results also suggested that eritadenine was resistant to high temperatures and its extraction yield could be enhanced by increasing the extraction temperature. Eritadenine was 2.2 folds more concentrated in the ExA than in the shiitake powder (Figure 3b), amounts that were only slightly lower than those described in other studies obtained with more complex extraction procedures.[25,58]

Lenthionine was detected in shiitake powder at similar levels than previously reported .[13,26] It was also noticed in higher concentrations in the ExA extracts (Figure 3b). Afterwards, only traces remained in ReA but probably the slight increase of temperature together with the high pressure utilized for SFE extraction was sufficient to degrade it being undetected in

364 the following extracted fractions. Lenthionine levels were drastically reduced when
365 extraction temperature was higher than 80 ^{o}C.[59,60] Thus, apparently pressurized extractions
366 made the compound more susceptible to degradation.

367 Ergosterol was mainly extracted with SFE (Table 2) obtaining a highly concentrated
368 ExB extract (88.7 mg/g) (Figure 3c). This result was not surprising since previous works
369 indicated that this lipophilic constituent that can be easily extracted with supercritical
370 CO_2.[21,61,62] Ergosterol in ExB represented 72 % of total sterols since the extract also included
371 other derivatives such as ergosta-7,22-dienol (18.5 mg/g), fungisterol (15.3 mg/g) and
372 ergosta-5,7-dienol (1.5 mg/g). The supercritical extraction was carried out using steel spheres
373 instead of sand as carrier material to facilitate the subsequent ReB separation for further
374 processing however, ergosterol yields were only slightly lower than usually obtained in
375 similar extractions using sea sand (8.9 and 11.8 % repectively).

376 Total phenolic compounds were mostly found in the water extracts. Obtained
377 fractions (ExA and ExC) contained respect. 3.0 and 2.5 fold more phenols than initially
378 detected in the mushroom powder. The second water extraction complemented the first one
379 leaving in the last residue a very low amount of these compounds (Figure 3c). These results
380 were in concordance with previous studies reporting that water achieved higher recoveries of
381 total phenolic compounds in comparison with organic solvents such as methanol.[16]

382 ***Biological activities of the obtained fractions***

383 The antioxidant activity of the obtained fractions was evaluated as their $ABTS^{+\bullet}$ and
384 $DPPH^{\bullet}$ scavenging capacities and as their ferric ion reducing power. Results indicated that
385 those fractions containing high concentrations of proteins, eritadenine and phenolic
386 compounds showed high antioxidant activities (Figure 4). Their levels were more than double
387 of the initial mushroom powder. ExA and ExC were the fractions with the lowest IC_{50} values

(respectively 0.05 and 0.06 mg/mL when using $ABTS^{+\bullet}$ and 0.15 and 0.17 mg/mL when using $DPPH^{\bullet}$) and the lowest EC_{50} for their reducing powder (0.26 and 0.28 mg/mL). According to their TEAC values, the obtained extracts and residues showed higher affinity for the $ABTS^{+\bullet}$ radical than for $DPPH^{\bullet}$. The use of different solvents might also influence the obtained results since less polar compounds were extracted when methanol was used to carry out the $DPPH^{\bullet}$ assay. The shiitake and ExA antioxidant activities were in concordance with previous studies[16, 63] where it was indicated that their antioxidant activities as radical scavengers were mainly due to the phenolic content and ergothioneine.[14] Similarly, the antioxidant activities noticed in the extracts also showed high correlations with their phenolic concentration (with $R^2 = 0.99$ for $ABTS^{+\bullet}$ and reducing power assays and 0.97 for $DPPH^{\bullet}$ assays).

Cholesterol lowering in serum can be achieved via several mechanisms but mainly by impairing of exogenous cholesterol absorption or inhibiting endogenous cholesterol biosynthesis. The hypocholesterolemic activity of the obtained fractions was evaluated as their ability to inhibit the key enzyme of the cholesterol biosynthetic pathway (HMGCR) since the capacity to impair cholesterol absorption was already evaluated elsewhere for similar extracts containing high ergosterol levels (as ExB) and high β-glucans and dietary fibers contents (as respect. ExC and ReC).[7,17] The initial shiikate powder showed a remarkable HMGCR inhibitory activity acting as pravastatin used as control (Figure 5). ExA showed similar inhibitory activity (98 %) than the mushroom indicating that the responsible compounds might be water soluble. However, the inhibitors were not completely separated with a single cold water extraction since ReA and ReB were still able to lower HMGCR activity (respectively 86 and 80 %). Perhaps, to further improve the extraction yield of HMGCR inhibitors serial extractions with cold water could be carried out although this might involve dilution of the other bioactive compounds. Increase of the water temperature for

extraction is not encouraged because after heat application, the inhibitory activity of the obtained fractions was drastically reduced to 31 and 24 % for respect. ExC and ReC, suggesting that the compounds responsible for the inhibition were thermolabile. This indication was also noticed in previous studies.[33] The ExB extract was also able to inhibit 59 % of the enzyme activity indicating that perhaps other compounds soluble in supercritical CO_2 (more lipophilic molecules) were also interfering with the HMGCR activity as was also previously noticed by Gil-Ramirez *et al*. (2013)[64] for SFE extracts obtained from *Agaricus bisporus.*

The hypotensive activity of the obtained fractions was evaluated as their capacity to inhibit the angiotensin converting enzyme. However, since it was previously reported that certain peptides were responsible compounds a more detailed study was carried out on the two extracts including larger amounts of proteins (ExA and ExC) by dividing them in 3 fractions of different molecular weights. The protein contents of fractions obtained from ExA were higher than those from ExC and within the fractions, lower molecular weight fractions contained less proteins, peptides and other N-containing compounds (Table 3). Fractions <10 kDa showed higher IC_{50} than higher MW fractions ranging from 0.45 mg/mL for the MW>10 kDa fraction from ExA up to 1.63 mg/mL for the MW<3 kDa fraction from ExC. These values were lower than those reported in similar studies where other mushrooms such as *Hypsyzigus marmoreus* (6.4 mg/mL)[11] or mycelia extracts from *Lactarius camphorates* (1.6 mg/mL)[12] were analyzed. A significant correlation between protein content and ACE inhibitory activity was found however, when IC_{50} values were expressed taking into account the amount of proteins detected, fractions with lower molecular weight displayed the largest inhibitory capacity. Therefore, these results were in concordance with previous studies carried out on close related species such as *Lentinula polychrous* where small peptides were pointed as responsible compounds of their hypotensive properties.[65]

Conclusions

The present study showed that bioactive compounds can be differentially extracted from *Lentinula edodes* fruiting bodies or by-products using a sequential extraction method. Extracts obtained with cold water (ExA) contained high levels of water soluble β-glucans, chitooligosaccharides and other carbohydrates that were pointed as potential responsible for the high HMGCR inhibitory activity showed in this extract. It also contained eritadenine with cholesterol lowering properties, lenthionine that can inhibit platelet aggregation, peptides with ACE inhibitory capacity and antioxidant phenols. Then, the residue after this extraction can be submitted to supercritical CO_2 extraction to obtain a fungal sterol-enriched fraction (ExB) with the ability of displacing cholesterol from dietary mixed micelles formed after digestion impairing its absorption. Afterwards, the remaining material can be submitted to hot water extraction yielding an extract (ExC) containing β-glucans with bile acid-binding capacities (able to interfere cholesterol absorption) and similar solubility than lentinan and a residue (ReC) including dietary fibres such as β-glucans and chitins that apparently, according to previous studies, modulate human microbiota reducing the risk of CVDs. Therefore, many biologically active compounds can be separated within different fractions from the same batch of shiitake mushrooms if the described methods are sequentially applied. Then, the obtained fractions will contain higher concentrations of the bioactive compounds than the mushroom itself and they will be transformed into more bioaccesible forms (*i.e.* dietary fibers will be more easily fermented by colonic microbiota activating hypocholesterolemic mechanisms or absorbed by M cells in the intestine).

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463 **Conflict of interest:** None.

464 **Notation**

465 ACE = Angiotensin-I Converting Enzyme

466 CVD = Cardiovascular disease

467 HMGCR = 3-hydroxy-3-methylglutaryl coenzyme A reductase

468 SFE = Supercritical fluid extraction

469 TEAC = Trolox equivalent antioxidant capacity

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Table 1. Total carbohydrates (TC), β-glucans (βG), chitins (CH), total proteins (PR), eritadenine (EA), lenthionine (LT), ergosterol (ER) and total phenolic compounds (PH) levels of shiitake fruiting bodies and two separated tissues (indicated values are w/w).

	TC (g/100g)	**βG (g/100g)**	**CH (g/100g)**	**PR (g/100g)**	**EA (mg/g)**	**LT (mg/g)**	**ER (mg/g)**	**PH (mg/g)**
Whole	40.67±0.65	29.32±1.04	6.03±0.29	13.42±0.22	1.43±0.13	0.15±0.01	2.40±0.02	9.27±0.05
Cap	39.72±2.42	29.89±1.55	5.96±0.87	13.81±0.12	1.13±0.01	0.13±0.01	2.24±0.04	9.68±0.50
Stipe	42.37±1.42	32.01±1.72	6.28±1.09	12.90±0.08	1.12±0.11	0.11±0.01	2.32±0.05	8.95±0.43

No significant differences ($P \leq 0.05$) were found between different samples for the same component.

Table 2: Distribution of bioactive compounds within the obtained fractions expressed as percentage of initial dry mushroom powder (taking into consideration the obtained yields).

	TC (g/100 g)	βG (g/100 g)	CH (g/100 g)	PR (g/100g SP)	EA (mg/g)	LT (mg/g)	ER (mg/g)	PH (mg/g)
SP	40.67±0.34^{a}	29.32±1.04^{a}	6.03±0.29^{a}	13.42±0.22^{a}	1.43±0.13^{a}	0.15±0.01^{a}	2.40±0.02^{a}	9.27±0.05^{b}
ExA	11.46±0.37^{d}	5.10±0.09^{d}	1.58±0.08^{d}	5.86±0.49^{b}	1.46±0.14^{a}	0.09±0.01^{b}	0.03±0.01^{e}	10.35±0.01^{a}
ReA	33.84±1.80^{b}	20.32±0.73^{b}	4.11±0.26^{b}	4.53±0.01^{c}	0.32±0.01^{bc}	0.01±0.01^{c}	2.12±0.11^{b}	4.84±0.20^{c}
ExB	n.d.^{e}	n.d.^{f}	n.d.^{f}	n.d.^{e}	n.d.^{c}	n.d.^{c}	0.79±0.01^{d}	0.04±0.00^{e}
ReB	34.07±0.94^{b}	21.17±0.24^{b}	3.66±0.10^{b}	4.36±0.04^{c}	0.41±0.03^{bc}	n.d.^{c}	1.08±0.01^{c}	4.33±0.10^{c}
ExC	9.06±0.31^{d}	3.53±0.65^{e}	0.86±0.03^{e}	1.63±0.01^{d}	0.57±0.03^{b}	n.d.^{c}	0.06±0.01^{e}	2.91±0.01^{d}
ReC	22.14±1.05^{c}	14.38±0.15^{c}	2.29±0.06^{c}	2.00±0.04^{d}	0.17±0.00^{c}	n.d.^{c}	1.01±0.11^{cd}	2.85±0.33^{d}

n.d. = not detected

$^{a\text{-}f}$Different letters denote significant differences ($P \leq 0.05$) between different samples for the same component.

Table 3: Total protein content (PR) and ACE inhibitory activity (IC_{50}) of the different molecular weight fractions obtained from ExA and ExC.

Extract	Fraction	PR (mg/g fraction)	IC_{50} (mg fraction/mL)	IC_{50} /PR (mg protein/mL)
ExA	MW >10 kDa	391.93±2.28[a]	0.45±0.01[c]	0.18±0.03[ab]
	10 > MW > 3 kDa	237.97±3.34[b]	0.91±0.08[bc]	0.22±0.02[a]
	MW < 3 kDa	18.10±1.28[f]	1.06±0.09[bc]	0.02±0.00[c]
ExC	MW >10 kDa	192.81±3.28[c]	0.74±0.07[c]	0.14±0.00[b]
	10 > MW > 3 kDa	87.77±2.28[d]	1.21±0.09[b]	0.11±0.01[c]
	MW < 3 kDa	35.49±1.09[e]	1.63±0.15[a]	0.06±0.00[c]

[a-f]Different letters denote significant differences ($P \leq 0.05$) between different samples for the same column.

Figure 1. Outline of the sequential extraction method utilized to obtain different extracts from shiitake mushrooms.

Figure 2. Extraction yields obtained after each step of the sequential extraction method expressed as percentage (w/w) referred to the initial amount of shiitake powder (SP). Different letters (a-f) showed statistical significance ($P \leq 0.05$) between different extractions.

Figure 3. Distribution of bioactive compounds within the extracts obtained from shiitake powder expressed as concentrations within each fraction. Levels of a) total carbohydrates, β-glucans, chitins and proteins, b) eritadenine and lenthionine and c) ergosterol and total phenolic compounds. Different letters (a-f) showed statistical significance ($P \leq 0.05$) between different samples for the same compound.

Figure 4: $ABTS^{+\bullet}$ and $DPPH^{\bullet}$ scavenging capacity and ferric ion reducing power of shiitake powder and the fractions obtained after the sequential extraction method expressed as their TEAC value. Different letters (a-f) showed statistical significance ($P \leq 0.05$) between different samples.

Figure 5: HMGCR inhibitory activity of shiitake powder and the fractions obtained after the sequential extraction method. Different letters (a-d) showed statistical significance ($P \leq 0.05$) between different samples.

Figure 1:

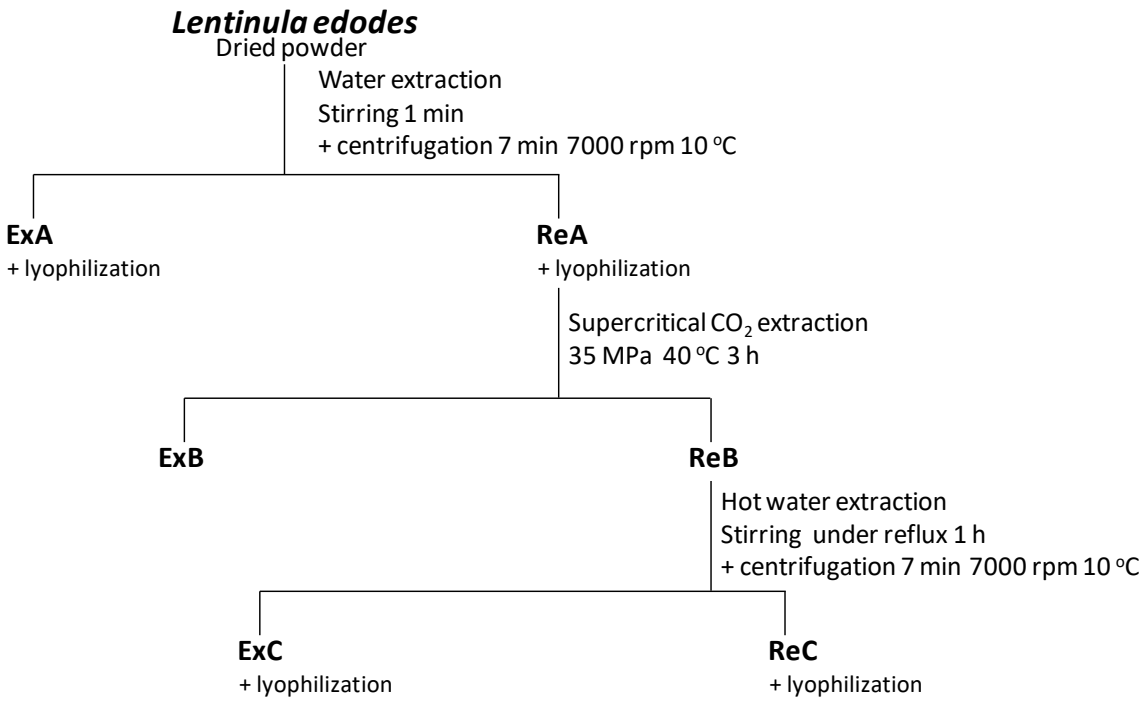

Lentinula edodes
Dried powder
Water extraction
Stirring 1 min
+ centrifugation 7 min 7000 rpm 10 °C
ExA
+ lyophilization
ReA
+ lyophilization
Supercritical CO_2 extraction
35 MPa 40 °C 3 h
ExB
ReB
Hot water extraction
Stirring under reflux 1 h
+ centrifugation 7 min 7000 rpm 10 °C
ExC
+ lyophilization
ReC
+ lyophilization

Figure 2:

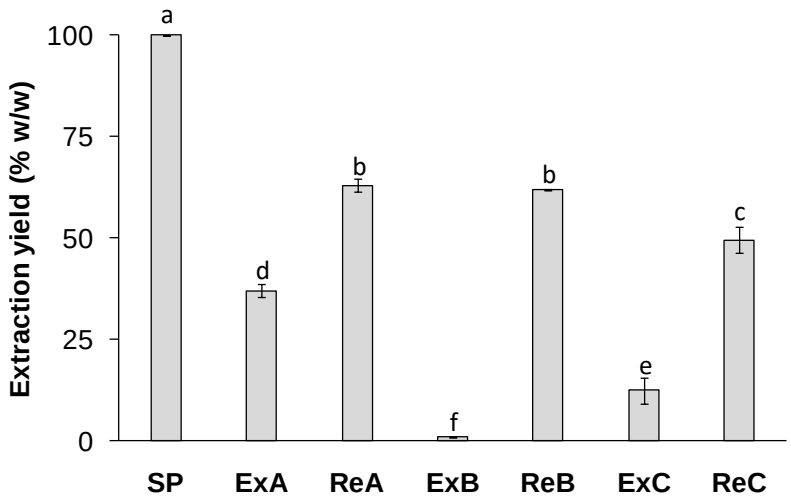
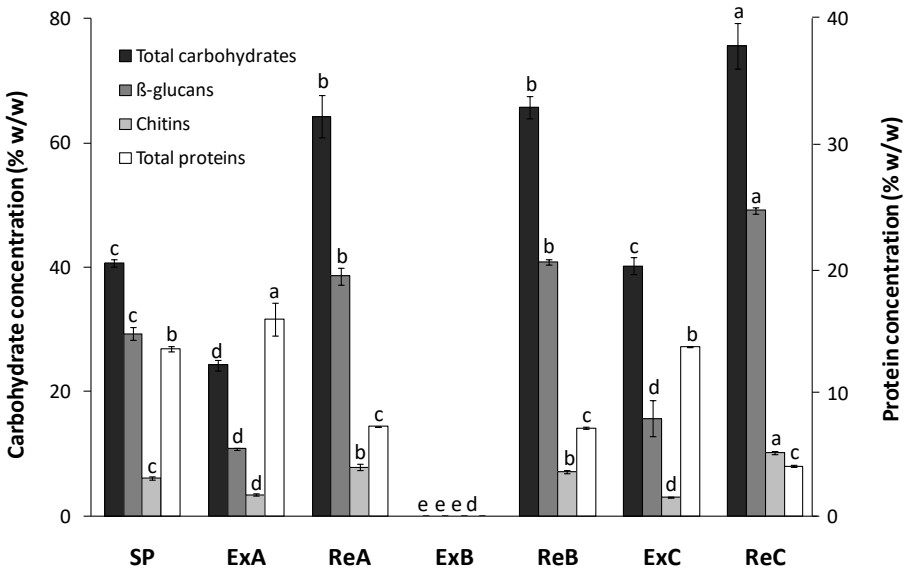
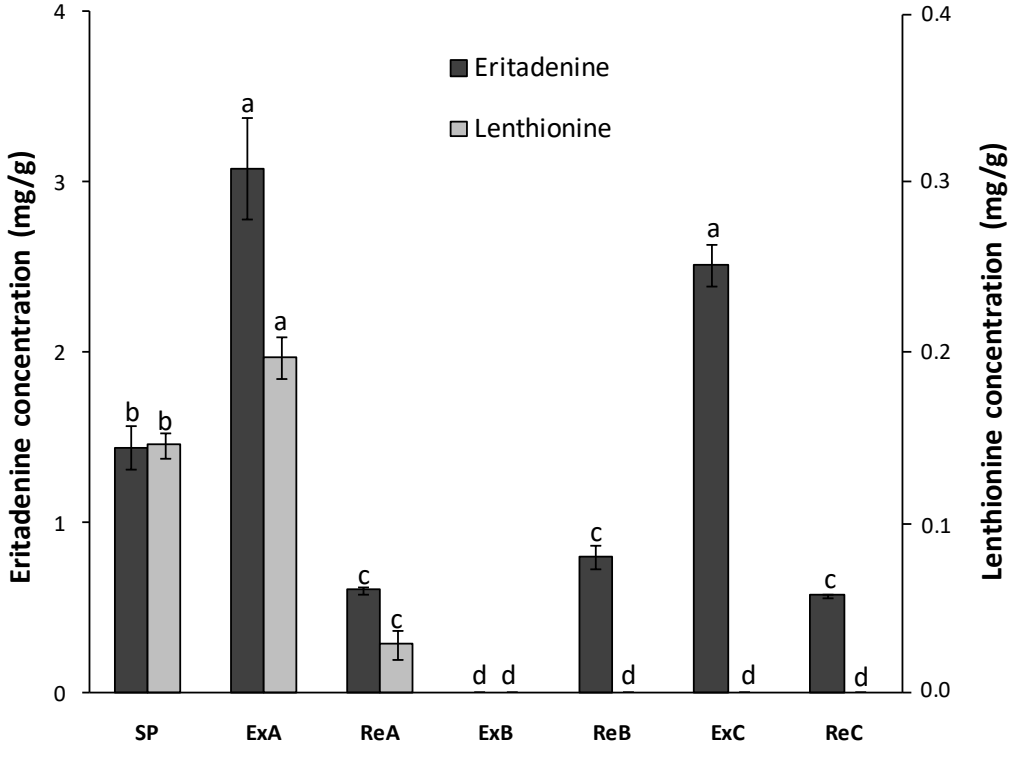

Extraction yield (% w/w)
100
75
50
25
0
a
d
b
f
b
e
c
SP
ExA
ReA
ExB
ReB
ExC
ReC

Figure 3:

a)


Total carbohydrates
ß-glucans
Chitins
Total proteins
Carbohydrate concentration (% w/w)
Protein concentration (% w/w)
0
20
40
60
80
0
10
20
30
40
SP
ExA
ReA
ExB
ReB
ExC
ReC

b)


Eritadenine
Lenthionine
Eritadenine concentration (mg/g)
Lenthionine concentration (mg/g)
0
1
2
3
4
0.0
0.1
0.2
0.3
0.4
SP
ExA
ReA
ExB
ReB
ExC
ReC

c)

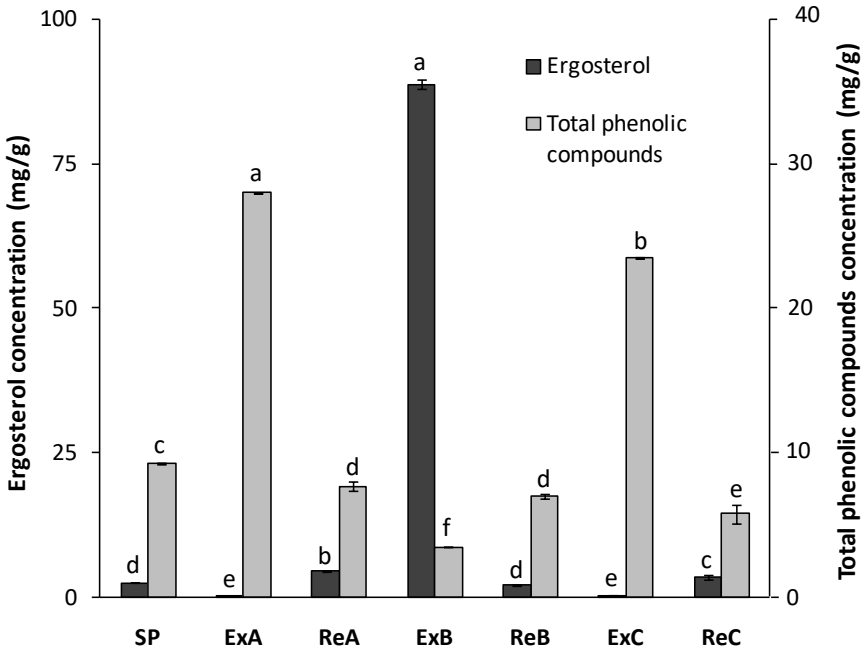

100
75
50
25
0
40
30
20
10
0
Ergosterol concentration (mg/g)
Total phenolic compounds concentration (mg/g)
Ergosterol
Total phenolic compounds
a
a
b
d
d
e
b
d
d
e
c
e
f
c
d
e
SP
ExA
ReA
ExB
ReB
ExC
ReC

Figure 4:

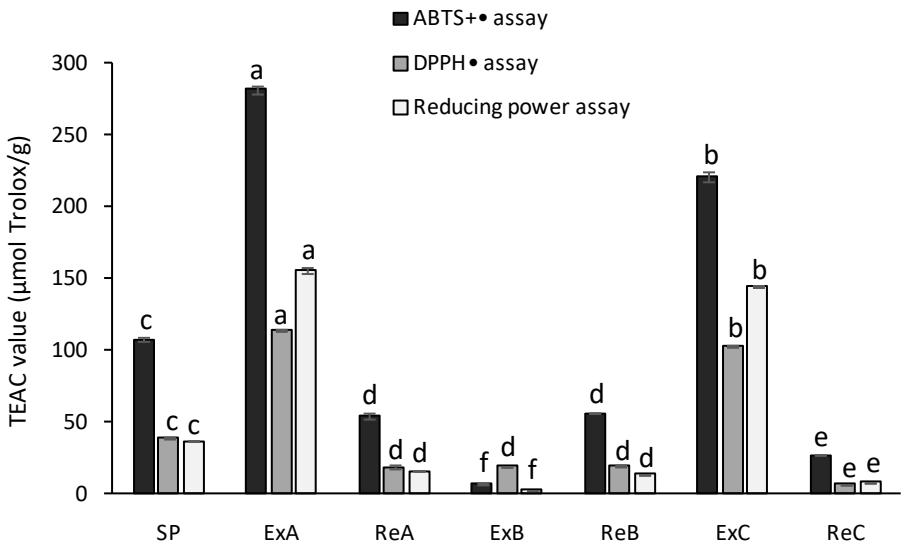
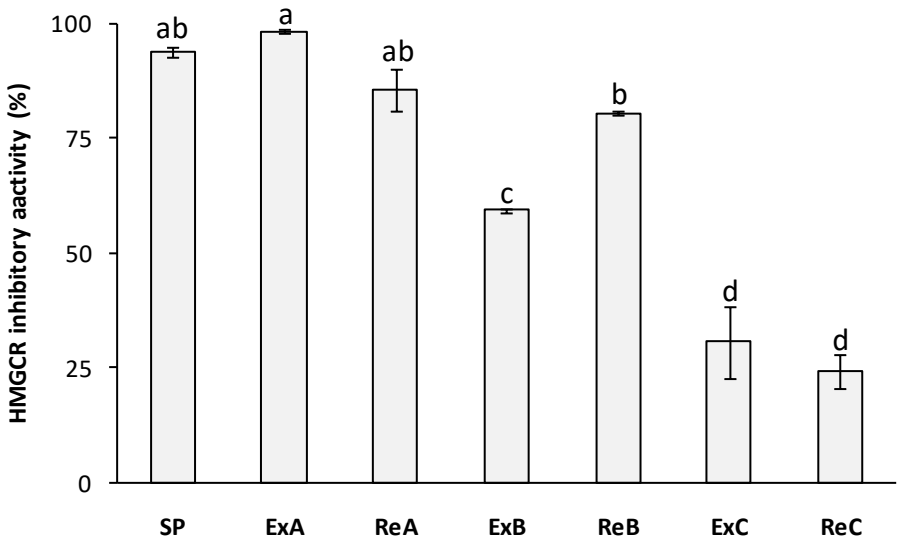

ABTS+• assay
DPPH• assay
Reducing power assay
TEAC value (µmol Trolox/g)
300
250
200
150
100
50
0
a
a
a
b
b
b
c
c
c
d
d
d
d
d
d
e
e
e
f
d
f
SP
ExA
ReA
ExB
ReB
ExC
ReC

Figure 5:


HMGCR inhibitory aactivity (%)
100
75
50
25
0
ab
a
ab
c
b
d
d
SP
ExA
ReA
ExB
ReB
ExC
ReC