EDIBLE MUSHROOMS AS POTENTIAL SOURCES OF NEW HYPOCHOLESTEROLEMIC COMPOUNDS

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ABSTRACT

Coronary heart disease (CHD) is the leading cause of death in the Western world after cancer according to World Health Organization. Many studies have established that high total-cholesterol levels and low-density lipoprotein (LDL) cholesterol levels are risk factors for CHD and mortality.

Many investigations have been carried out to explore the possibility of increasing components which have hypocholesterolemic effects in the diet. Two particular groups of substances phytosterols and β-glucans gained much attention in the last decade and there are already commercialized functional food products supplemented with plant sterols and/or derivatives (sterol-esters, stanols, etc) and specific polysaccharides mainly obtained from cereals which are able to inhibit the absorption of exogenous cholesterol.

Edible mushrooms are good sources of phytosterol-like structures such as ergosterol, fungisterol and other derivatives since they were present in all mushrooms because they are constitutive compounds of the hyphae membranes. On the other hand, edible mushrooms contained polysaccharides and depending on the specie, they showed high levels of β-glucans. Apart from these compounds, some mushroom species included certain molecules that were different than lovastatin (since statins were not detected) able to impair the synthesis of endogenous cholesterol by inhibiting the HMGCoA reductase (3-hydroxy-3-methyl-glutaryl CoA reductase) the key enzyme in the cholesterol metabolism.

Keywords: cholesterol, statins, glucans, sterols, HMGCoA reductase

INTRODUCTION

Maintenance of cholesterol homeostasis is one of the major issues in the human body since it is a key constituent of the cell membranes. Thus, if the molecule is not obtained from diet liver synthesizes it by a specific metabolic pathway. When cholesterol enters the lumen of the small intestine it is coming from 3 different sources: diet, bile and intestinal epithelial sloughing. Nowadays in industrialized countries, the average daily intake is approximately 300 – 500 mg. Bile provides 800 mg – 1200 mg cholesterol per day to the intraluminal pool. The turnover of intestinal mucosal epithelium is approximately 300 mg cholesterol per day. The synthesized cholesterol can reach ca. 1000-1600 mg per day. When an excess of exogenous cholesterol is absorbed and reach the liver, it induces several regulation effects such as inhibition of cholesterol biosynthetic pathway and of the LDL-R (low density lipoproteins-receptor) gene expression [1, 2].
Nutritionists have given wide range of dietary recommendations (fruit, vegetables, fish etc.) but with limited success. Only the novel foods products (yogurt, breakfast cereals etc.) offered in the market claiming hypocholesterolemic effects have got a little higher acceptance by consumers. At present there are two types of functional foods recognized by EFSA as able to reduce the risk of CVD because of their ability to reduce cholesterol absorption [3, 4]. One is those products including plant sterols (phytosterols) or derivatives (sitostanol esters etc.). Apparently, intaking of 1.5 g/day of these compounds reduced LDL-cholesterol in hypercholesterolaemic patients by 10 and 15% within 3 - 4 weeks [5]. The other type of functional foods is those containing β-glucans, mainly obtained from cereal products and able to reach 15% cholesterol reduction [6].

Both functional products are able to lower cholesterol in serum by reducing its absorption. However, it has been shown that in subjects who were administered β-glucan, the cholesterol biosynthetic pathway was stimulated compared with control subjects [6]. Thus, in order to increase treatment efficiency, it could be necessary to combine inhibitors of the cholesterol absorption with inhibitors of the cholesterol synthesis.

Edible mushrooms are good sources of phytosterol-like structures such as ergosterol, fungisterol and many other derivatives. The major fungal sterol, ergosterol (9.61-1.28 mg/g dw), is abundant in all mushrooms species since it is a constitutive compound of the hyphae membranes and it is known as a vitamin D₂ (ergocalciferol) precursor [7 - 9]. These molecules might act as plant phytosterols and reduce cholesterol absorption by displacement of the molecule from the dietary mixed micelles formed during intestinal digestion.

Beside oat bran, pectin, guar gum etc. edible mushrooms also contains β-glucans such as lentinan from shiitake, schizophyllan from splitgill, grifolan from maitake mushrooms, α- and β-glucans from sun, reishi and oyster mushrooms, glucuronoxylomannans from tree-ear and white jelly-leaf mushroom etc. [10] and some of them were able to effectively lower serum cholesterol levels [11 – 13]. Apparently, the viscous and gel-forming properties of these compounds could lower the cholesterol absorption by inhibiting the formation of micelles in the small intestine and perhaps they might also interact with the bile acids similarly as explained for bran β-glucans, leading to an increase in faecal bile acids excretion and increasing of hepatic conversion of cholesterol into bile acids [12].

However, if cholesterol is not obtained from the diet, it enhances the de novo synthesis in the liver. Statins or vastatins are the most potent drugs available for reducing plasma low density lipoproteins (LDL)-cholesterol concentrations [14]. According to previous reports, several oyster mushroom strains showed lovastatin (mevinolin) a compound able to lower cholesterol levels by inhibiting the 3-hydroxy-3-methyl-glutaryl CoA reductase (HMGCoA-red), a key-enzyme in the cholesterol metabolism [15]. However, other compounds such as lanosteroids, ganoderols etc., obtained from other mushrooms are also able to perform such an inhibition [11].

Thus, in this work a preliminary screening of these potentially active compounds is carried out as an attempt to design new functional foods based on mushrooms extracts able to effectively reduce the cholesterol levels in serum by impairing both the synthesis and the absorption of cholesterol.

**MATERIAL AND METHODS**

**Biological material and samples preparation.** Mushroom strains used in this investigation were Cantharellus cibarius (Fr.), Agaricus bisporus L. (Imbach), Pleurotus ostreatus (Jacq.Ex Fr.) Kummer, Lentinus edodes S. (Berkeley), Boletus edulis (Bull. Ex Fr.), Amanita caesarea (Scop. Ex Fri.) Pers. Ex Schw., Lactarius deliciosus (Fr.), Lyophyllum Shimeji (Kawam.), Agrocybe aegerita (Briganti) Singer, Ganoderma lucidum (Curtis) P.Karst., Craterellus
cornucopioides (L. Ex Fr.) Pers, Marasmius oreades (Bolt. Ex Fr.) Fr., Pleurotus eryngii (D.C. Ex Fr.) Quel, Lepiota procera (Scop. Ex Fr.) Singer, Agaricus blazei Murill ss. (Heinem), Amanita ponderosa Malençon & R. Heim, Grifola frondosa (Dicks.) Gray and Flammulina velutipes (Curt. Ex Fr.) Singer.

Fruiting bodies from wild mushrooms were purchased on season from the local market in Madrid (Spain) and the cultivated strains were harvested from the cultivation rooms at CTICH (Autol, Spain) facilities. Fruiting bodies were dehydrated and ground into fine powder as described by Ramirez-Anguiano et al. (2007) [16]. Dried mushroom powders were stored at -20ºC until further use.

**Reagents.** Ascorbic acid and KOH were obtained from Sigma-Aldrich. Ethanol was purchased from Panreac, 2,6 di-tert-butyl-p-cresol (BHT) from Fluka, ergosterol from Alfa Aesar GmbH & Co KG (Germany), pravastatin, lovastatin, simvastatin and atorvastatin from Cinfa (Spain).

**Determination of sterols levels in mushrooms.** Freeze-dried mushroom powders (0.2 g) and 0.3 g of ascorbic acid were mixed with 15 ml of 11.5% KOH in ethanol:water (55:45 v/v) and vigorously stirred for 15 min at 80ºC. Afterwards, the mixture was cooled down and 10 ml of 0.01% (w/v) BHT in hexane were added. The mixture was shacked during 2 min and left at room temperature (5 min) until complete separation of the phases. Organic fraction was collected and 5 ml BHT solution were added to the aqueous fraction for a second extraction. Both organic phases were pooled together in a round bottom flask and evaporated on a rotary vacuum extractor at 30ºC until dryness. Dry extracts were dissolved in a CHCl₃:MeOH (2:1 v/v) solution including hexadecane as internal standard and submitted to GC analysis.

Mushroom extracts were injected into a GC column (Zebron ZB-5 30m x 0.25 mm, 0.25 µm, Phenomenex, France) from a CP-3380 gas chromatography (Varian, Spain) with a flame ionization detector (FID). Split was set 1:10. The injector was set at 260ºC, the detector at 350ºC and the oven temperature was maintained at 60ºC for 1 min, then increased with a rate of 40ºC/min until a final temperature (310ºC) that was maintained during 30 min (modification of the method proposed by Teichman et al. (2007)[8]).

A standard curve of ergosterol was used to develop and validate the GC method (linearity, LOD, LOQ, precision and reproducibility were determined using standardized protocols) and to quantify ergosterol and derivatives.

**Determination of β-glucans levels in mushrooms.** The beta-glucan content of the selected mushroom powders (50 mg) was determined according to the protocol described at the user’s manual of the Megazyme assay for mushroom and yeast β-glucan determination (Megazyme, Barcelona).

**Determination of HMGCoA-red inhibitors in mushrooms.** Mushroom powders (50 mg/ml) were dissolved in methanol, water or combined mixtures. Suspensions were shacked in a Vortex for 1 min and centrifuged at 12000 g 2 min. Supernatants (10 µl) were applied into a 96 wells-plate according to the user’s manual of the HMG-CoA Reductase Assay Kit (Sigma, Madrid). Absorbance at 340 nm was monitored at 37ºC using a microplate reader (Tecan Group Lt, Switzerland).

Several extraction methods were tested to isolate statins such as mixture of 200 mg mushroom powder with methanol: water (1:1 v/v), methanol or water and 20 mM phosphate buffer (pH 7.7): acetonitrile (1:1 v/v) following the procedures before described for lovastatin determination [15, 17, 18]. Depending on experiment, mushroom extracts and lovastatin solutions were centrifuged (12000 g 2 min) and the obtained supernatants filtered through 0.45 µm filters (Millipore). Filtrates were injected into a Sep-Pak® C18 cartridge (Waters)
Mushroom extracts showing higher HMGCoA-red inhibitory activity (20 μl) and statins were injected in an HPLC system (Agilent) equipped with a column (Zorbax SB-C18 0.3x150mm, 5μm particle size, Agilent) and developed using an isocratic mixture of CH$_3$CN: 0.5% CH$_3$COOH (60:40) and a at a flow of 1 ml/min. Peaks were detected with a Diode array detector and identified comparing the spectra and retention times with those of a few statins. The same samples and lovastatin solutions (20µL) were also injected in an LC-MS (Agilent 6410A Triple Quadrupole LC/MS system coupled with an Agilent 1200 Series Rapid Resolution (RRLC) system) with a C18 column (ACE 3 C18-AR, 150 x 4.6mm particle size 3μm) and developed on isocratic conditions using CH$_3$CN: 0.5% CH$_3$COOH (60:40) as mobile phase and a flow rate of 0.5 ml/min. The column eluent was introduced into the electrospray ionization source. The nebulizing gas flow-rate was 9mL/min, drying gas temperature was 350 °C and the capillary voltage was 3500 V. The samples responses from the column were monitored in the positive as well as in the negative ionization mode with full scan from (m/Z: 90-1200).

RESULTS AND DISCUSSION

Ergosterol-derivatives in mushrooms. Ergosterol (ergosta-5,7,22-trien-3β-ol) and its derivatives are compounds structurally similar to plant phytosterols however, only a few reports studied their influence on the cholesterol metabolism. Ergosterol was pointed as a potent agonist for liver X receptor (a factor involved in the regulation of cholesterol homeostasis) and as inducer of ABC-transporters expression (promoters of the active efflux of cholesterol and plant sterols from the enterocyte into the intestinal lumen for excretion). It was also described as a potent C24-reductase inhibitor, an enzyme which catalyzes the reduction of the double bond at C-24 in the cholesterol-biosynthesis pathway [20].

![Figure 1: GC chromatogram of the unsaponifiable fraction of Amanita caesarea. (a) ergosterol, (b) ergosta-7,22-dienol, (c) ergosta-5,7-dienol, (d) fungisterol.](image)

Ergosterol was the major sterol found in all the analyzed samples (Figure 1) except for G. lucidum which showed similar concentrations of ergosterol and ergosta-7,22-dienol. The distribution of ergosterol derivatives was strain dependent (Table 1). Some mushroom species such as C. cibarius and C. cornucopioides showed almost exclusively ergosterol, other species showed more ergosta-5,7-dienol than fungisterol (ergosta-7-enol) (L. shimeji, P. ostreatus), other lacked one or two of the derivatives or presented similar concentrations of the three identified ergosterol-derivatives.
Table 1: Sterols concentration (mg/g dw) in several mushroom species.

<table>
<thead>
<tr>
<th>Mushroom specie</th>
<th>Ergosta-5,7,22-trien-3-ol (ergosterol)</th>
<th>Ergosta-7,22-dienol</th>
<th>Ergosta-5,7-dienol</th>
<th>Ergosta-7-enol (fungisterol)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cantharellus cibarius</em></td>
<td>2.61</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2.61</td>
</tr>
<tr>
<td><em>Agaricus bisporus</em></td>
<td>3.06</td>
<td>0.65</td>
<td>0.67</td>
<td>0.67</td>
<td>5.05</td>
</tr>
<tr>
<td><em>Pleurotus ostreatus</em></td>
<td>3.75</td>
<td>0.79</td>
<td>0.98</td>
<td>0.51</td>
<td>6.04</td>
</tr>
<tr>
<td><em>Lentinula edodes</em></td>
<td>5.51</td>
<td>0.40</td>
<td>ND</td>
<td>0.43</td>
<td>6.34</td>
</tr>
<tr>
<td><em>Boletus edulis</em></td>
<td>5.69</td>
<td>1.21</td>
<td>0.93</td>
<td>0.87</td>
<td>8.71</td>
</tr>
<tr>
<td><em>Amanita caesarea</em></td>
<td>3.81</td>
<td>1.05</td>
<td>1.15</td>
<td>1.09</td>
<td>7.09</td>
</tr>
<tr>
<td><em>Lactarius deliciosus</em></td>
<td>1.60</td>
<td>0.36</td>
<td>ND</td>
<td>ND</td>
<td>1.96</td>
</tr>
<tr>
<td><em>Lyophyllum shimeii</em></td>
<td>4.64</td>
<td>ND</td>
<td>1.55</td>
<td>0.68</td>
<td>6.87</td>
</tr>
<tr>
<td><em>Agrocybe aegerita</em></td>
<td>5.11</td>
<td>ND</td>
<td>1.01</td>
<td>ND</td>
<td>6.12</td>
</tr>
<tr>
<td><em>Ganoderma lucidum</em></td>
<td>0.69</td>
<td>0.59</td>
<td>0.17</td>
<td>0.25</td>
<td>1.70</td>
</tr>
<tr>
<td><em>Craterellus cornucopioides</em></td>
<td>0.79</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.79</td>
</tr>
<tr>
<td><em>Marasmius oreades</em></td>
<td>3.85</td>
<td>0.54</td>
<td>0.59</td>
<td>0.57</td>
<td>5.55</td>
</tr>
<tr>
<td><em>Pleurotus eryngii</em></td>
<td>1.40</td>
<td>0.20</td>
<td>0.25</td>
<td>0.22</td>
<td>2.06</td>
</tr>
<tr>
<td><em>Leptota procera</em></td>
<td>2.57</td>
<td>0.67</td>
<td>ND</td>
<td>0.64</td>
<td>3.88</td>
</tr>
<tr>
<td><em>Agaricus blazeii</em></td>
<td>1.73</td>
<td>1.06</td>
<td>0.60</td>
<td>0.75</td>
<td>4.13</td>
</tr>
<tr>
<td><em>Amanita ponderosa</em></td>
<td>1.65</td>
<td>ND</td>
<td>0.60</td>
<td>0.62</td>
<td>2.87</td>
</tr>
<tr>
<td><em>Grifola frondosa</em></td>
<td>3.24</td>
<td>0.57</td>
<td>ND</td>
<td>0.61</td>
<td>4.42</td>
</tr>
</tbody>
</table>

ND: Not detectable

Ergosterol concentrations in the selected strains ranged from 0.7 up to 5.7 mg/g dw being *B. edulis* the specie with the highest levels and *C. cornucopioides* the specie with the lowest concentration of total sterols. These results were in concordance with values previously reported for some of the strains for instance, *B. edulis* was also pointed as the mushroom with the largest ergosterol content with concentrations estimated between 9.61-4.89 mg/g depending on authors, *C. cibarius* showed 2.78-3.04 mg/g, *A. bisporus*, *P. ostreatus* and *L. edodes* 7.8-4.4 mg/g. Other mushroom species such as *Suillus granulatus* showed 7.02 mg/g ergosterol and 0.8 mg/g fungisterol (ergosta-7-enol), *Russula cyanoxantha* and *Clitocybe nebularis* contained 1.28 and 1.04 mg/g fungisterol too [8, 9, 21, 22]. However, no previous information concerning the ergosterol content was found for other mushroom species indicated in this study such as for instance *L. procera*, *M. oreades*, *A. aegerita*, *L. shimeji* etc.

**β-Glucans in mushrooms.** At present, fungal polysaccharides are the subject of several studies because their specific carbohydrate composition and structure appears to confer many important biological activities as antitumour, antioxidant, antiviral, immunomodulatory activities etc [10]. Many of the polysaccharides responsible for those activities are β-glucans including hypocholesterolemic activities [6, 13, 23].

β-Glucans were also quantified in the same mushroom species than above described and similarly, their β-glucan content was strain dependent (Figure 2). Some of them showed a high β-glucan concentration such as for instance *G. Lucidum*, *P. ostreatus*, *L. edodes*, *A. aegerita* and *L. deliciosus*. However, mushrooms such as *L. procera*, *A. blazeii* and *A. bisporus* showed low levels compared to the others.
These values were higher than those previously reported by few publications from the same research group [24 – 26] since the described β-glucan contents ranged from 0.14 to 0.53 mg/100 mg in similar strains such as Pleurotus pulmonarius, P. ostreatus, P. eryngii, B. edulis, A. aegerita and L. edodes. Differences between results could be due to values expression since in their first publication they express their results on ‘dry matter’ basis and later for similar values they indicate that they were referred to their ‘edible portion’ which for raw mushrooms could be considered as ‘fresh weight’. Moreover, in their β-glucan extraction procedure fresh fruiting bodies were utilized while dry mushroom powder was used to determine the β-glucan content of the samples presented in Figure 2.

A more recent publication estimating the total β-glucan levels by a completely different method indicated concentrations between 2.6 - 13.4 mg/100mg dw for A. bisporus, F. velutipes, L. edodes, P. ostreatus and P. eryngii [27] and Lee et al. [28] ranged the β-glucan contents from 3.2 mg/100mg for F. velutipes to 33.5 mg/100mg for G. frondosa with other intermediate values for other species including Pholliota nameko. Those values are more in concordance with the presented results.

**HMGCoA-red inhibitors in Oyster mushrooms.** Nowadays, there are many in vivo evidences indicating the capability of Pleurotus spp. fruiting bodies to lower cholesterol levels in serum [29, 30]. Apparently, this activity could be partially due to the presence of lovastatin, a statin detected in mycelia culture broths as well as in mushroom fruiting bodies in all the developmental stages and tissues [15, 17, 18].

A few P. ostreatus strains were screened for HMGCoA-red inhibitors using different extraction procedures such as a mixture of methanol:water (1:1 v/v), water or methanol with not or overnight incubation at 30ºC and applying different extract concentrations.

According to previous publications, overnight incubation (30ºC) of the fruiting bodies with methanol:water (1:1) was the best method to extract lovastatine from fresh Oyster mushrooms [15, 17, 18]. When the dry mushroom powders were tested, the three selected P. ostreatus strains showed significant HMGCoA-red inhibitory activity (54.4, 37.7 and 18.8%) and their inhibitory activity was increasing with increasing extracts concentrations (Fig. 3). However, no significant differences were found when the extracts were freshly prepared and applied or after overnight incubation.
Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products (ICMBMP7) 2011

Figure 3: HMGCoA-red activity in the presence of several Pleurotus ostreatus extracts prepared under different conditions. Pravastatin was used as positive inhibitory activity.

When 100% methanol or water were tested as solvents no significant inhibitory activity was found in the methanol extracts but for some strains, the water extracts showed a remarkable HMGCoA-red inhibitory activity (Fig. 3). Extraction in 20 mM phosphate buffer (pH 7.7) showed slightly higher HMGCoA-red values than water and its mixture with acetonitrile did not change the value indicating that water was the best solvent to extract HMGCoA-red inhibitors.

Mushroom samples and statin standards were injected in HPLC-DAD and developed with two different mobile phases, the one reported by Gunde-Cimerman & Cimerman [15] and one including acetonitrile and 0.5% acetic acid. The later method showed narrower peaks than the previously reported and proper separation of the 4 selected statins and their hydroxy acid forms therefore it was further utilized to detect and quantify statins in mushrooms (Figure 4).

Figure 4: HPLC chromatograms at 240 nm of a) Pleurotus ostreatus concentrated extract and b) a mixture of statins including pravastatin (R.T. 1.4 min), atorvastatin (R.T. 3.3 min), lovastatin (R.T. 9.9 min) and simvastatin (R.T. 14.4 min).

Pleurotus ostreatus water, methanol:water (with positive HMGCoA-red inhibitory activity) or methanol extracts yielded no detectable peak at the retention time of lovastatin. Neither compatible peak was detected when the samples were treated to generate lovastatin-hydroxy acid form. Only when the samples were concentrated using the SPE (Solid phase extraction) column, two peaks were observed at 8.9 and 9.9 min with similar spectra than lovastatin since they both showed a maximum at 240 nm (Figure 5).
Figure 5: UV-VIS spectra of a) peak eluting at 8.9 min and b) 9.9 min from the *P. ostreatus* extract and c) lovastatin spectrum (9.9 min).

Nevertheless, since many different compounds could also show similar UV-VIS spectra at that maximum, a standard of lovastatin and a *P. ostreatus* extract were injected into a LC-MS in order to determine and compare their masses.

Figure 6: UV-VIS and mass spectra of a) lovastatin (eluting at 8.2 min) and b) of *P. ostreatus* (peak with R.T. 8.2 min).

Lovastatin injected in an LC-MS showed a single peak with a retention time of 8.2 min and a single mass peak of 405.1 undoubtedly corresponding to the 404.55 gram/mol assigned by the literature (Figure 6a). However, the peak from the *P. ostreatus* extract eluting at similar R.T. showed a mass of 522.3 indicating that they were different compounds. Moreover, none of the other detectable peaks showed a mass similar to neither lovastatin nor other statins.
CONCLUSION
Mushrooms fruiting bodies or their extracts might be considered as a new source of compounds with potential hypocholesterolemic activity because they are rich in ergosterol-derivatives, β-glucans and HMGCoA-red inhibitors. Since not a single mushroom strain showed the highest levels of the three type of compounds, a mixture of a few of them could be used to prepare bioactive supplements to functionalize foods potentially able to reduce levels of cholesterol in serum.

Although lovastatin was not detected in the studied oyster mushrooms, water extracts showed remarkable HMGCoA-red inhibitory activity. Therefore, further investigations are at the present being carried out to optimize these supplements and to identify the compounds responsible for the HMGCoA-red inhibitory capacity observed.

ACKNOWLEDGEMENTS
The research was supported by the European Union within the 7th framework programme (7FM-PEOPLE-2009-IGF project 251285), by the ALIBIRD-CM S2009/AGR-1469 and ADER 2010-1-ID-0096 regional programs from respectively the Community of Madrid and La Rioja (Spain) and by the AGL2010-21537 national R+D program from the Spanish Ministry of Science and Innovation.

REFERENCES


