

**Screening of edible mushrooms and extraction by pressurized  
water (PWE) of 3-hydroxy-3-methyl-glutaryl CoA reductase  
inhibitors**

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## ABSTRACT

The methanol/water and particularly the water extracts obtained from 26 mushroom species were able to inhibit the 3-hydroxy-3-methyl-glutaryl CoA reductase (HMGCR) activity to different extent (10 to 76%). Cultivated mushrooms such as *Pleurotus* sp. and *Lentinula edodes* were among the strains which showed higher HMGCR inhibitory capacities. Their inhibitory properties were not largely influenced by cultivation parameters, mushroom developmental stage or flush number. The HMGCR inhibitory activity of *L. edodes* was concentrated in the cap excluding the gills while in *P. ostreatus* it was distributed through all the different tissues. A method to obtain aqueous fractions with high HMGCR inhibitory activity was optimized using an accelerated solvent extractor (ASE) by selecting 10.7 MPa and 25°C as common extraction conditions and 5 cycles of 5 min each for *P. ostreatus* fruiting bodies and 15 cycles of 5 min for *L. edodes* suggesting that the potential HMGCR inhibitors are different in the two selected mushrooms.

**Keywords:** cholesterol, HMGC $\alpha$ reductase, *Pleurotus ostreatus*, *Lentinus edodes*, accelerated solvent extraction (ASE)

## 1. INTRODUCTION

Coronary heart disease (CHD) is the leading cause of death in the Western world after cancer according to the World Health Organization. Many studies have established that high total-cholesterol and low-density lipoprotein (LDL) cholesterol levels are risk factors for CHD and mortality. Several *in vivo* studies have demonstrated the ability of certain edible mushrooms to lower cholesterol levels in serum. Species such as *Pleurotus* spp. (Opletal et al., 1997; Bajaj et al., 1997; Bobek&Galbavy, 1999; Schneider et al., 2011), *Agaricus bisporus* (Jeong et al., 2011), *Ganoderma lucidum* (Berger et al., 2004), *Lentinula edodes*, *Grifola frondosa*, *Flammulina velutipes* (Fukushima et al., 2001), *Auricularia auricular* and *Tremella fuciformis* (Cheung, 1996), among others (Roupas et al., 2012), have been investigated in animals and human studies.

Apparently, the hypocholesterolemic effect of the mushroom fruiting bodies and several types of their extracts is reached by different mechanisms of action such as impairing dietary cholesterol absorption or inhibiting the endogenous cholesterol metabolism (Guillamón et al., 2010). Mushrooms are rich in chitin (dietary fibre) and specific  $\beta$ -glucans which might inhibit cholesterol absorption by increasing the faecal excretion of bile acids and reducing the amount of serum LDL-cholesterol (Guillamón et al., 2010; Chen & Seviour, 2007).

Eritadenine (an adenosine analogue alkaloid) is another compound isolated from *Lentinula edodes* (shiitake mushroom) which is able to lower cholesterol levels. This molecule inhibits S-adenosylhomocysteine hydrolase and modifies the hepatic phospholipid metabolism (Chibata et al., 1969, Sugiyama et al., 1995, Yamada et al., 2007).

According to previous reports, oyster mushrooms (*Pleurotus* spp.) contained lovastatin, a compound able to lower cholesterol levels inhibiting 3-hydroxy-3-methyl-glutaryl CoA reductase (HMGCR) (Gunde-Cimerman et al., 1993; Gunde-Cimerman&Cimerman, 1995), the key-enzyme in the cholesterol metabolism. Statins are the most potent drugs available for

1 reducing plasma low density lipoproteins (LDL) in cholesterol concentrations (Shitara&  
2 Sugiyama, 2006). However, other reports found no detectable statins levels in *Pleurotus* sp.  
3 fruiting bodies although high HMGCR inhibition activities were recorded (Schneider et al.,  
4 2010; Gil-Ramirez et al., 2011). Other compounds (obtained from *Ganoderma lucidum*) were  
5 also described as being able to impair the proper function of the enzyme (Berger et al., 2004).

6 Thus, in this work a preliminary screening of HMGCR inhibitors was carried out using  
7 several edible mushroom species and varieties. The screening was also performed in  
8 cultivated mushrooms harvested from cultivation rooms with different cultivation parameters  
9 in an attempt to define the conditions required for the synthesis of the effective inhibitors.  
10 Once the mushrooms varieties containing higher HMGCR inhibitory activity were defined,  
11 they were submitted to pressurized solvent extractions (or accelerated solvent extractions,  
12 ASE) in order to optimize environmentally friendly and GRAS methods able to obtain fungal  
13 fractions with high HMGCR inhibitory activity to further functionalize foods with potentially  
14 hypocholesterolemic properties (Chen et al., 2011).

## 16 2. MATERIAL AND METHODS

### 17 2.1 Biological material and samples preparation

18 Mushroom strains used in this investigation were *Lentinus edodes* S. (Berkeley), *Cantharellus*  
19 *cibarius* (Fr.), *Lactarius deliciosus* (Fr.), *Boletus edulis* (Bull. Ex Fr.), *Pleurotus ostreatus*  
20 (Jacq.Ex Fr.) Kummer, *Agaricus bisporus* L. (Imbach), *Amanita caesarea* (Scop. Ex Fri.)  
21 Pers. Ex Schw., *Morchella esculenta* (Pers Ex Amans), *Agaricus blazei* Murill ss. (Heinem),  
22 *Grifola frondosa* (Dicks.) Gray, *Ganoderma lucidum* (Curtis) P.Karst., *Flammulina velutipes*  
23 (Curt. Ex Fr.) Singer, *Pleurotus eryngii* (D.C. Ex Fr.) Quel, *Lyophyllum shimeji* (Kawam.),  
24 *Morchella conica* (Pers.), *Agrocybe aegerita* (Briganti) Singer, *Auricularia judea* (Bull. Ex  
25 St.Amans) Berck, *Amanita ponderosa* Malençon & R. Heim, *Craterellus cornucopioides* (L.

Ex Fr.) Pers, *Marasmius oreades* (Bolt. Ex Fr.) Fr., *Lepiota procera* (Scop. Ex Fr.) Singer.,  
*Pholiota nameko* (T. Itô) S. Imai, *Calocybe gambosa* (Fr.) Donk, *Hydnum repandum* (Linné  
Ex Fr.), *Cantharellus lutescens* (Pers.), *Pleurotus pulmonarius* (Fr.) Quel.

Fruiting bodies from wild mushrooms were purchased from a local market in Madrid (Spain).  
The cultivable strains were grown in cultivation rooms with automatic control of cultivation  
parameters (temperature, R.H., CO<sub>2</sub>) at CTICH (Centro Tecnológico de Investigación del  
Champiñón de La Rioja, Autol, Spain) or at the cultivation facilities of some mushroom  
growers belonging to the La Rioja's mushroom association using commercially available  
substrates depending on the mushroom specie to cultivate. Fruiting bodies were harvested at  
the usual developmental stage prior to commercialization except in those experiments when  
the effect of the developmental stage was studied. The recorded parameters are described in  
Table 1.

Complete fruiting bodies or their separated tissues were immediately frozen, freeze-dried,  
ground and sieved until the particle size smaller than 0.3 mm as described in Ramirez-  
Anguiano et al. (2007) and stored at -20°C until further use.

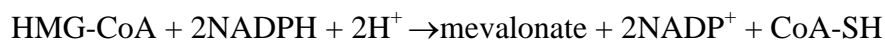
To extract the potential inhibitors from the spores allowing the breaking down of the spore  
walls, they were first treated with methanol as described by Gunde-Cimerman et al. (1993).

## **2.2 Determination of HMGCoA-red inhibitors in mushrooms**

Mushroom powders (50 mg/ml) were mixed with water, methanol/water (1:1 v/v) or  
methanol. Suspensions were shaken in a Vortex for 1 min and centrifuged at 12000 rpm  
(8,854 x g) for 2min (Eppendorf mini-spin, Madrid, Spain) according to the user's manual.  
Supernatants were used as source of HMGCR inhibitors.

HMGCR activity was measured using the commercial HMG-CoA Reductase Assay (Sigma,  
Madrid) according to the user's manual. The assay is based on the spectrophotometric

measurement of the decrease in absorbance at 340 nm, which represents the oxidation of NADPH by the catalytic subunit of HMGCR in the presence of the substrate HMG-CoA according to the reaction:



Mushroom supernatants (20 µl) were applied into a 96 wells-plate and their absorbance change was monitorized at 37°C using a microplate reader (Tecan Group Lt, Männedorf, Switzerland).

Pravastatin was utilized as a control for positive inhibition. Other control samples were prepared in each assay by substituting the mushroom extract by the same solvent solution utilized in the extract. These controls were considered as 100% activity and tested samples were referred to them as percentage of inhibition or activity. Assays were performed in duplicate.

### **2.3 Pressurized water extractions to obtain fractions with HMGCR inhibitory activity**

Mushroom powders (1g) were mixed with sea sand (Sigma, Madrid, Spain) (4g) and submitted to pressurized solvent extraction at 1500 psi (10.68 MPa) using an accelerated solvent extractor (ASE) (Dionex, ASE 350, Sunnyvale, CA, USA). Several parameters such as extraction time or cycles number and temperature were changed in order to optimize the extraction method to obtain fractions with high HMGCR inhibitory activity.

Obtained fractions were immediately frozen, lyophilized and stored at -18°C until further analysis. Extracted dry matter content was measured to calculate the extraction yields.

### **2.4 Statistical analysis**

One way analysis of variance (ANOVA) was performed using a Statgraphics® Plus 3.1 for Windows software (Statistical Graphics Corporation, Rockville, MD, USA). The mean comparison test used was Fisher's least significant differences procedure (LSD).

### 3. RESULTS AND DISCUSSION

#### 3.1 Screening of mushrooms species as a source of HMGCR inhibitors

Several wild and cultivated mushroom species were submitted to water and methanol/water (1:1, v/v) extraction since both type of solvents were previously described as able to extract compounds with HMGCR inhibitory activity (Gil-Ramírez et al., 2011). Methanol was also tested with a few strains but no interesting HMGCR inhibitory activity was found in any of the selected samples. Results indicated a wide variability within the different mushroom species (Figure 1). The water extracts of 8 species showed no significant HMGCR inhibitory activity and many showed inhibitory activities from 10 to 50%. However, strains such as *Pleurotus* sp., *Cratharellus cornucopiodes*, *Amanita ponderosa* and particularly *Lentinula edodes* showed inhibitory capacities ranging from 52 up to 76%. In most of the cases except for *Agaricus bisporus* and *Cantharellus lutescens*, the water extracts showed higher inhibitory activity than the methanol/water extracts. Surprisingly, *A. bisporus* methanol/water extracts showed almost 2 fold more capacity than the water extracts.

No similar distribution was found between genera belonging to the same family, for instance, within the Pleurotaceae family (*P. ostreatus*, *P. pulmonarius* and *P. eryngii*) two species showed similar and high HMGCR inhibitory capacities but *P. eryngii* showed only 20.7% inhibitory capacity. On the contrary, the Agaricaceae family included two species (*A. bisporus* and *A. blazeii*) with low inhibition in their water extracts while *L. procera* was able to inhibit almost 50% of the enzyme. The Cantharellaceae family (*C. lutescens*, *C. Cibarius* and *C. cornucopioides*) as well as other families such as Marasmiaceae (*M. oreades* and *L.*

*edodes*), Morchellaceae (*M. conica* and *M. esculenta*) and Amanitaceae (*A. caesarea* and *A. ponderosa*) included species with no, middle and high inhibitory capacities.

### **3.2 Influence of the cultivation parameters on the HMGCR inhibitory activity of cultivated mushrooms**

The water extracts from cultivated mushrooms such as *Pleurotus* sp. and *L. edodes* showed very interesting inhibitory activities and their growth can be more easily controlled than wild mushrooms. Environmental parameters usually influence the presence/absence of many compounds therefore; a more detailed study was carried out in those cultivated mushrooms in order to determine the effect of the cultivation parameters in their HMGCR inhibitory activities.

Substrates were inoculated with commercial spawns from several *P. ostreatus* and *L. edodes* varieties in a minimum of three independent trials but performed under similar conditions. After cultivation, the obtained fruiting bodies from the first flush were harvested and their water extracts analyzed. Results showed no significant differences between Fungisem K15 and Sylvan HK35 but they were significantly higher in Fungisem K15 than the other analyzed varieties (Figure 2a). Similar results were obtained when the different *L. edodes* spawns were studied (Figure 2b); significant differences were found between Sylvan 4312 and Mycelia 3710 or Le Lion L8 but the standard deviation observed within similar trials suggested that other parameters different than variety are involved in the HMGCR inhibitory activity observed.

Therefore, other parameters were investigated such as mushroom grower, automatic control of cultivation conditions (or traditional cultivation system), presence of bacterial blotch infection, pests (flies, mosquitoes etc.), good or poor quality fruiting bodies or mushroom



picked from places close to a *Trichoderma* sp. spot. However, no correlation was found within the detected HMGCR inhibitory activities and any of the indicated parameters.

### **3.3 Developmental and tissue distribution of the HMGCR inhibitory activity of cultivated mushrooms**

*P. ostreatus* and *L. edodes* fruiting bodies from the same cultivation trial were harvested at different developmental stages including from primordia until cap was open and gills exposed (mature). The harvesting was repeated in the second and third flush. No significant differences were observed between flushes or developmental stages in the HMGCR inhibitory activity of the water extracts obtained from *P. ostreatus* mushrooms. In *L. edodes* mushrooms a slight decrease of the inhibitory capacity was observed with the increase of the flush number but no significant differences were found between young or mature fruiting bodies. This suggest that the compounds responsible for the HMGCR inhibitory activity are present through the whole sporophore life cycle and that they might be different between the two studied mushroom strains. This view was further supported by the fact that compounds potentially responsible for the inhibitory activity showed a different tissue distribution within the fruiting bodies. The water extracts obtained from different tissues of *P. ostreatus* showed similar HMGCR inhibitory activity indicating a homogeneous distribution (Figure 3a) though the whole body while in the selected *L. edodes* variety the inhibitory activity was concentrated in the dermis and epidermis excluding the gills and the stipe (Figure 3b).

Worth to take into consideration was the HMGCR inhibitory activity levels found in the discarded parts of the stipes, this part correspond to the lower stipe which is bound to the mycelium and the substrate, at the base of the fruiting body. Usually, this part of the mushroom is cut during harvesting because it might contain traces of substrate or it is

deformed and not commercialized. The HMGCR inhibition values of the discarded by-product obtained from *L. edodes* varied depending on the strain since Amycell 4012 showed no HMGCR inhibitory activity but Sylvan 4312 showed a 37.1% inhibition, higher values than the 10.8% inhibition found in the discarded stipe of *P. ostreatus*.

### **3.4 Pressurized water extraction of the cultivated mushrooms to obtain fractions with HMGCR inhibitory activity**

Pressurized water extractions are economically sustainable processes that are increasingly being developed as a response to the demand (by the food and other industries) of environmentally clean extraction processes to produce new extracts or compounds with a potential use as functional ingredients or nutraceuticals (Sun et al., 2012). Water is a non-toxic and naturally occurring solvent that can replace organic solvents when used at higher temperatures and pressures. Accelerated solvent extraction using water has been used to extract *i.e.* polysaccharides with different structures and biological activities from edible mushrooms (Lo et al., 2007; Di et al., 2003) and they are, at the present, being scaled up for industrial applications (Pronyk & Mazza 2009).

*P. ostreatus* and *L. edodes* fruiting bodies were submitted to extraction with pressurized water in order to obtain mushroom extracts with high HMGCR inhibitory activity to be in the future potentially used as hypocholesterolemic ingredient to functionalize foods. Thus, several parameters such as extraction temperature, time and cycles number were changed in order to optimize the extraction method.

A range of temperatures was studied from 25 up to 200°C and the obtained dry matter increased largely with the temperature, being 200°C the best condition to extract almost 60 and 80% of the material from *P. ostreatus* and *L. edodes*, respectively (Figure 4a). However,

when the HMGCR inhibitory capacity of the extracted fractions was measured, the increase of temperature was detrimental suggesting that the compounds responsible for the inhibition were thermolabile (Figure 4b). No significant differences were found between extractions at 25 or 50°C in both mushroom strains neither between these extractions from *L. edodes* and extractions with plain water however, water extraction of *P. ostreatus* showed a significantly higher inhibitory activity (80.1%) than its ASE extractions. Thus, extraction temperature was fixed at 25°C and other ASE parameters were modified in order to improve extraction yields and inhibitory activities.

Extractions carried out by selecting 5 cycles of 1 min each yielded almost half of the dry matter than longer extraction times from *P. ostreatus* mushrooms however, no significant differences were observed for *L. edodes* within 1 to 10 min; apparently all the water soluble compounds at 25°C are easily extracted after 5 cycles of 5 min from both strains (Figure 5a). The ASE extracts obtained from both mushroom strains after 5 cycles of 1 to 10 min each showed high HMGCR inhibitory capacity while longer extractions showed a slightly lower inhibitory activity, suggesting that the compounds potentially responsible for the inhibition might be unstable if extracted for 75 min total extraction time (Figure 5b). The *P. ostreatus* extract obtained after 5 cycles of 1 min showed similar inhibitory capacity than longer extraction times, however, less dry matter was obtained pointing the 5 min extraction as the best extraction time, thus, the optimal number of cycles was further studied.

Increase of the extraction cycles (of 5 min each at 25°C) from 1 to 15 cycles increased the extracts yields almost 3 fold for both mushroom strains (Figure 6a). However, for *P. ostreatus*, the ASE extracts obtained after 5 cycles showed the highest inhibitory activity while no significant differences were observed within the HMGCR inhibitory capacity of all the ASE extracts from *L. edodes* obtained independently of the number of cycles (Figure 6b). These results suggested once more that the compounds responsible of the inhibitory capacity

might be different in both mushrooms since 5 cycles should be the optimal to extract the potentially enzyme inhibitors from *P. ostreatus* while higher cycles number can be used to extract higher amount of *L. edodes* extracts. But, both inhibitors are easily extracted with water and their HMGCR inhibitory activity is eliminated at temperatures where the typical protein denaturalization takes place suggesting that they might share similar chemical nature and it could include a proteic part in its structure. Actually, preliminary studies are being carried out suggesting that the inhibitors might be proteoglucans.

#### 4. CONCLUSION

Several wild and cultivated mushroom species contain water soluble compounds potentially capable of inhibiting the key enzyme of the cholesterol metabolism using *in vitro* test. Two of the cultivated strains, Oyster and Shiitake mushrooms, were particularly interesting. Their higher or lower inhibitory capacity was probably dependent on specific requirements since no specific influence of the commercial variety, cultivation parameters or developmental stage of the mushroom was observed. Tissue distribution within the fruiting bodies was different suggesting the presence of different compounds with HMGCR inhibitory activity in both species. The potential inhibitory compounds could be easily extracted by using pressurized water extractions at 10.68 MPa and 25°C, 5cycles of 5 min for *P. ostreatus* and up to 15 cycles of 5 min for *L. edodes*. Thus, by using the optimized extraction methods, higher quantities of the potential inhibitors in will be extracted for further identification of the responsible compounds. The purification steps will have to be separately carried out for each mushroom since the responsible compounds might be different.

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- 1 **Table 1** Cultivation parameters recorded and correlated with the HMGCR inhibitory activity
- 2 values obtained within the analyzed samples (*P. ostreatus* and *L. edodes*).

Parameter	Different type of samples
	5 different commercial varieties per specie:
Commercial spawn	<i>P. ostreatus</i> K15 Fungisem, HK35 Sylvean, K40 Fungisem, S-300 Mispaj and H9 Gurelan and <i>L. edodes</i> 4312 Sylvan, S05 Mispaj, 3710 Mycelia, Le Lion L8 and 4012 Amycel.
Substrate	Pasteurized straw (for <i>P. ostreatus</i> ) and sterilized/pasteurized sawdust (for <i>L. edodes</i> )
Disease influence	Healthy, brown blotch, trichoderma, pests
Developmental stage	Primordia, small, medium, large
Flush number	First, second, third and fourth flush
Mushroom growers	CTICH and 12 mushroom growers
Modern controlled chambers	Yes/No

3

**Legends of the figures:**

**Figure 1:** HMGCR inhibitory activities of several mushrooms extracts obtained in water or methanol/water (1:1, v/v).

**Figure 2:** HMGCR inhibitory activities of water extracts from the fruiting bodies obtained by cultivating different commercial spawns from a) *Pleurotus ostreatus* and b) *Lentinula edodes*.

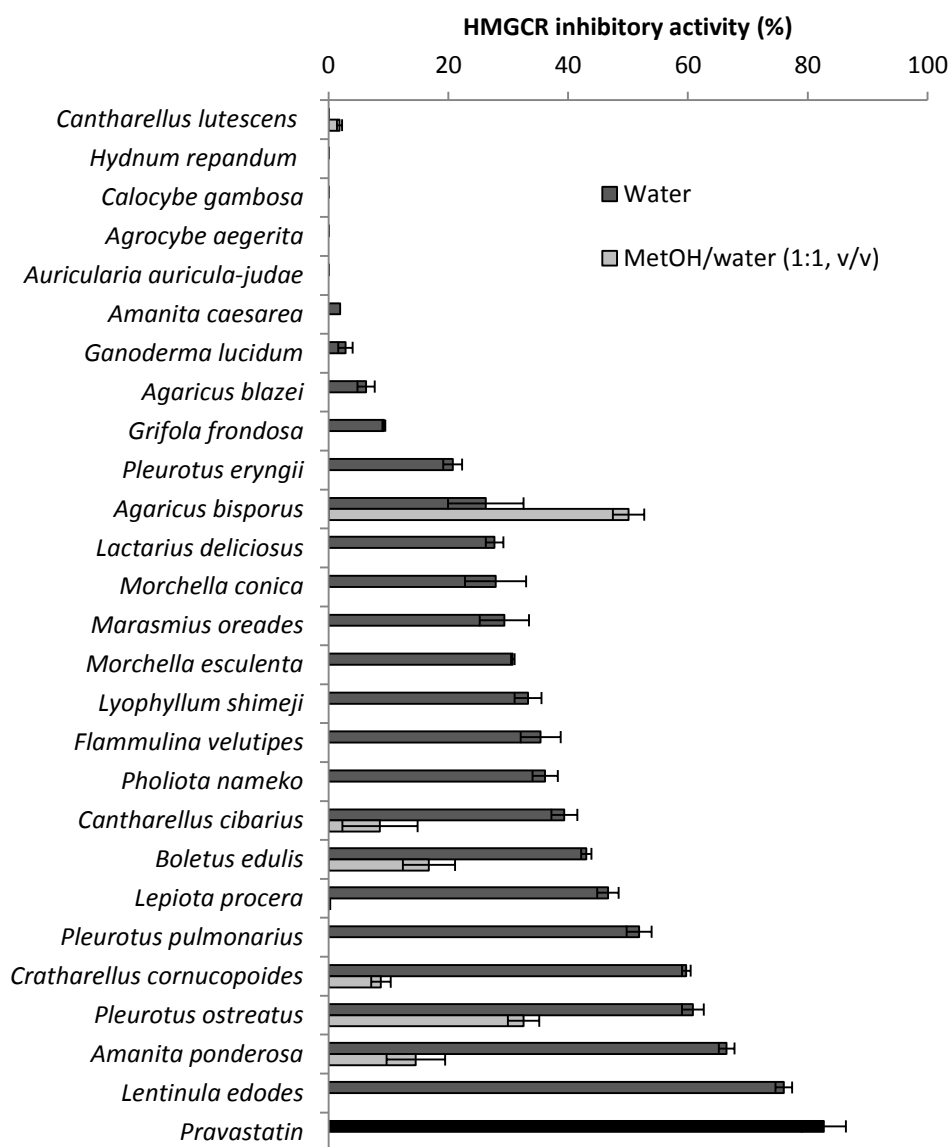
**Figure 3:** HMGCR inhibitory activities of water extracts from different tissues from fruiting bodies from the first flush of a) *Pleurotus ostreatus* Sylvan HK35 and b) *Lentinula edodes* Amycell 4012.

**Figure 4:** a) ASE extraction yield and b) HMGCR inhibitory activities of *Pleurotus ostreatus* and *Lentinula edodes* fractions obtained after 5 cycles of 5 min each at different temperatures.

**Figure5:** a) ASE extraction yield and b) HMGCR inhibitory activities of *Pleurotus ostreatus* and *Lentinula edodes* fractions obtained at 25°C but at different extraction times per cycle (5 cycles).

**Figure6:** a) ASE extraction yield and b) HMGCR inhibitory activities of *Pleurotus ostreatus* and *Lentinula edodes* fractions obtained at 25°C but after a different number of cycles (5 min each).

1 **Figure 1:**

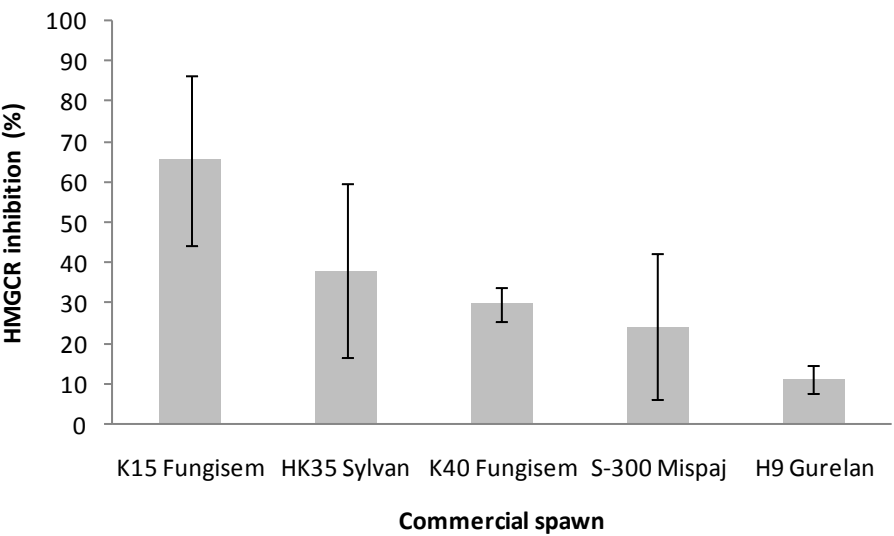


2

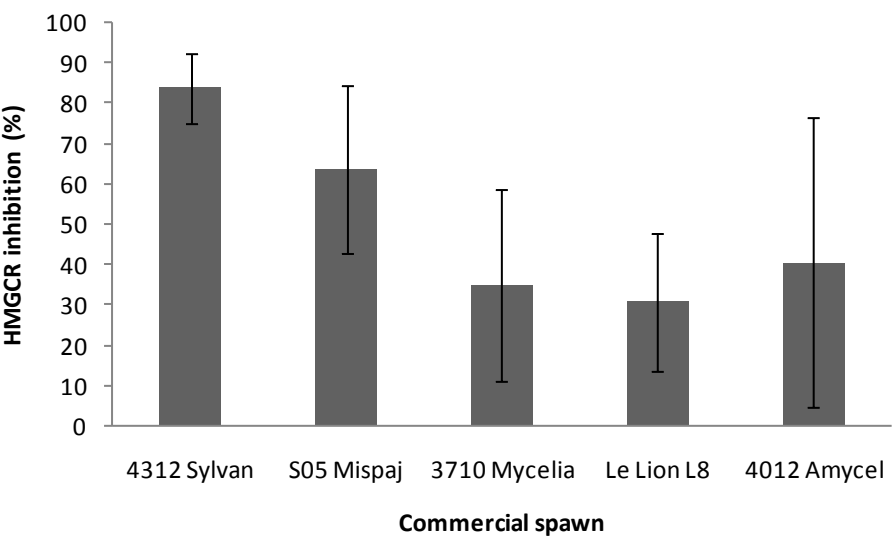
3

**Figure 2:**

a)

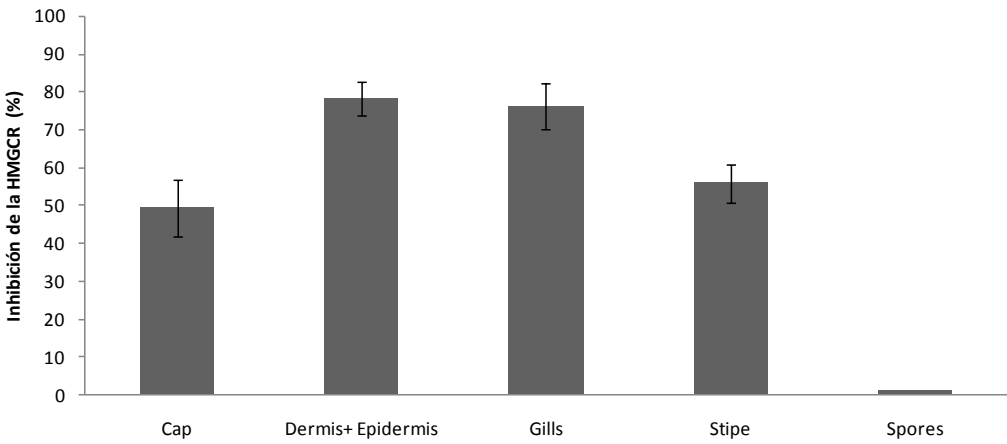


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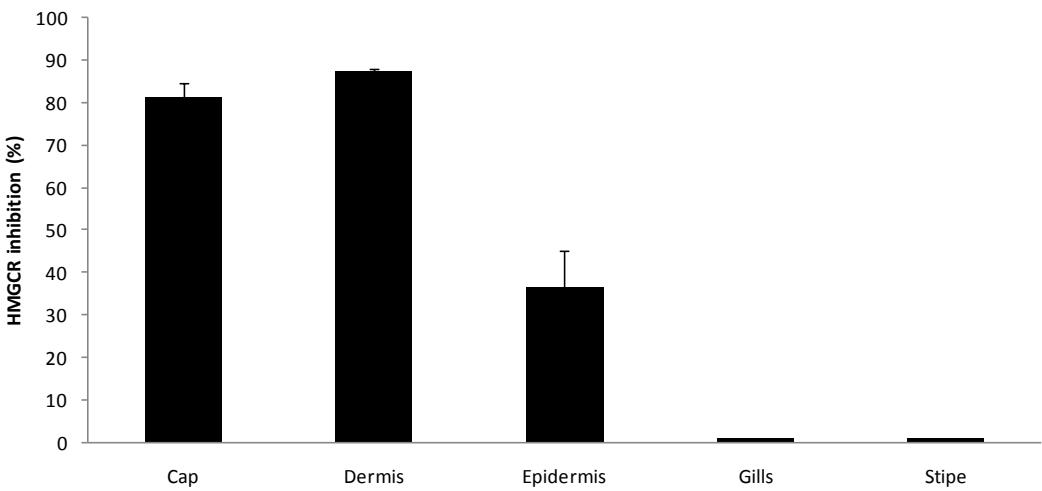


**Figure 3:**

a)

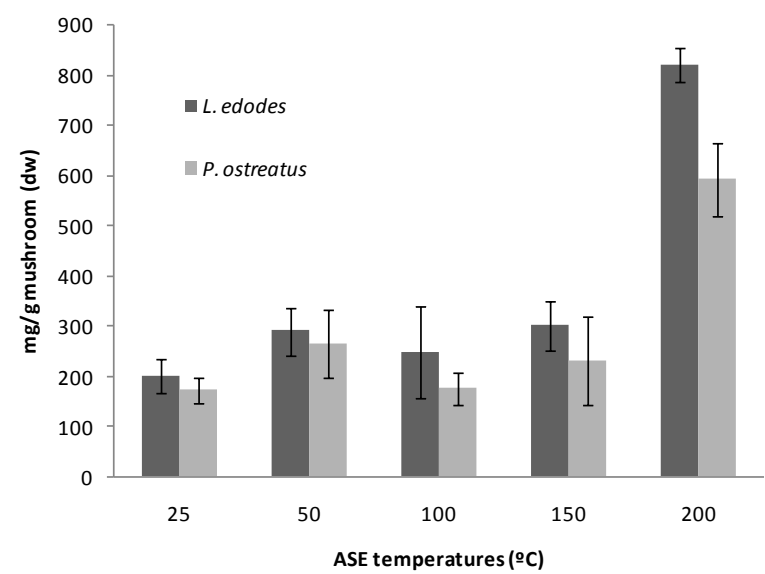


b)

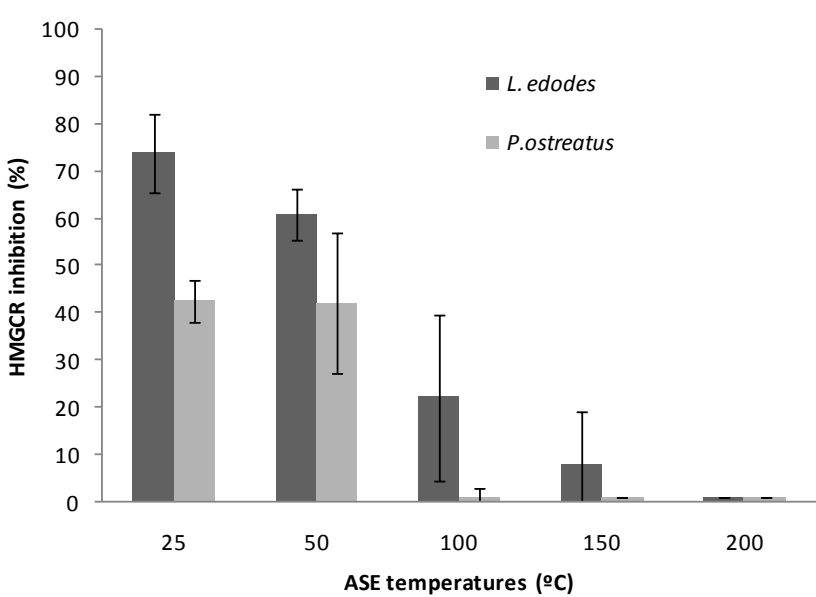


**Figure 4:**

a)

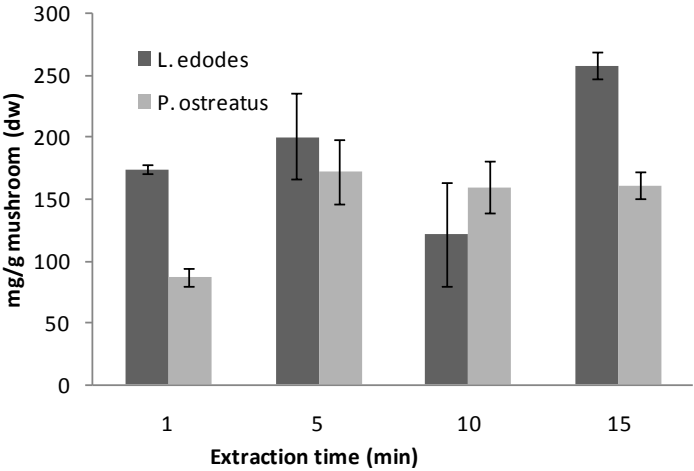


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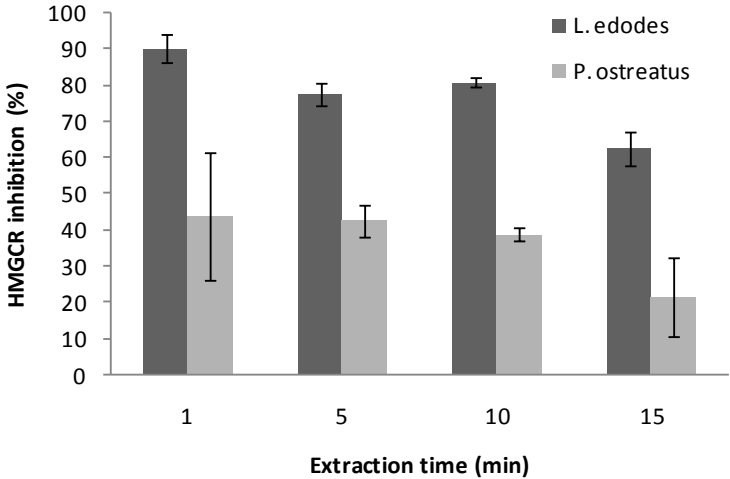


**Figure5:**

a)

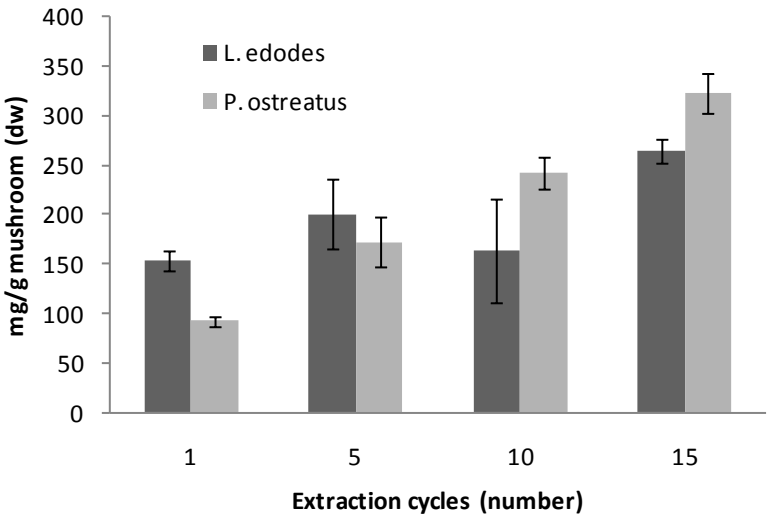


b)



**Figure6:**

a)



b)

