

**Effect of traditional and modern culinary processing, bioaccessibility, biosafety  
and bioavailability of eritadenine, a hypocholesterolemic compound from edible  
mushrooms**

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## 14    **Abstract**

15            Eritadenine is a hypocholesterolemic compound that was found in several mushroom  
16 species such as *Lentinula edodes*, *Marasmius oreades*, *Amanita caesarea* (1.4, 0.7 and 0.6  
17 mg/g dw, respectively). It was synthesized during all developmental stages being present in  
18 higher concentrations in the skin of shiitake fruiting bodies. When submitted to traditional  
19 cooking, grilling followed by frying were more adequate methodologies than boiling or  
20 microwaving to maintain its levels. Modern culinary processes such as texturization (with agar-  
21 agar), spherification (with alginate) also interfered with its release. Grilling and gelling using  
22 gelatin enhanced eritadenine bioaccessibility in an *in vitro* digestion model. An animal model  
23 (where male and female rats were administrated 21 and 10 mg/kg animal/day of eritadenine)  
24 indicated that in taking of the compound was safe under those concentrations, it reached the  
25 liver and reduced the atherogenic index (TC/HDL) in rat sera. Thus, it might be used to design a  
26 functional food.

## 1. Introduction

Edible mushrooms are consumed worldwide due to their particular flavors and nutritional values. Their consumption is also encouraged because of their health promoting properties *e.g.* their ability to lower cholesterol levels in serum. Therefore, certain mushroom species could be used as starting material to design functional foods. They contain fungal sterols and  $\beta$ -glucans that can impair dietary cholesterol absorption<sup>1</sup> and other molecules that affect the biosynthesis of endogenous cholesterol.<sup>2,3</sup> They also synthesize other compounds such as eritadenine that can also indirectly influence serum LDL levels.<sup>4</sup>

Eritadenine ((2(R),3(R)-dihydroxy-4-(9-adenyl)butanoic acid), also named lentysine or lentynacin, is an adenosine analog derived from the secondary metabolism that was firstly isolated from shiitake mushrooms (*Lentinula edodes*).<sup>5</sup> Several studies demonstrated that its hypocholesterolemic activity in mice and rats was related to its ability as S-adenosyl-L-homocysteine hydrolase (SAHH) inhibitor. Eritadenine modulated hepatic phospholipid metabolism by decreasing the phosphatidylcholine (PC)/phosphatidylethanolamine (PE) ratio. However, its precise mechanism of action is not yet fully elucidated<sup>4,6,7</sup> and therefore, certain safety risks might arise if food products are functionalized by adding eritadenine-enriched extracts as hypocholesterolemic ingredients.

Another aspect to take into consideration is the fact that edible mushrooms (or those functionalized foods) are not usually consumed raw but they are submitted to culinary treatments before ingestion. These processes, usually involving heat (boiling, grilling, etc.), might modulate the concentrations of the fungal bioactive molecules that are assimilated.<sup>8,9</sup> Moreover, novel culinary procedures that are nowadays in trend, presenting surprising dishes or food products (gelling, spherification, etc.) to potentiate consumer interest, might influence their levels too since they include food additives in their formulations (hydrocolloids, thickeners, etc.). These additives could directly interact with the bioactive ingredients modifying their bioaccessibility. Later on, the bioactive compounds that survive the culinary

treatments might be further modified during mastication, stomach and/or intestinal digestions influencing their absorption levels at the human intestine.<sup>10</sup> However, not many studies have been carried out to evaluate the influence of all these processes on bioactive compounds such as eritadenine.

Therefore, a screening through several edible mushrooms was carried out to find an interesting eritadenine source. Different fruiting body tissues and developmental stages were also investigated. Then, shiitake mushrooms were submitted to traditional and modern culinary processings to evaluate their effect on eritadenine stability. Afterwards, the effect of digestion on eritadenine bioaccessibility was studied using an *in vitro* digestion model and its toxicity, bioavailability and hypocholesterolemic effect were evaluated with *in vivo* animal experiments.

## **2. Materials & Methods**

### *2.1. Biological material*

Mushroom species such as *Lentinula edodes* S. (Berkeley), *Lactarius deliciosus* (Fr.), *Boletus edulis* (Bull. Ex Fr.), *Pleurotus ostreatus* (Jacq.Ex Fr.) Kummer, *Agaricus bisporus* L. (Imbach), *Amanita caesarea* (Scop. Ex Fri.) Pers. Ex Schw., *Cantharellus tubaeformis* (Schaeff) Quel, *Ganoderma lucidum* (Curtis) P.Karst., *Lyophyllum shimeji* (Kawam.), *Morchella conica* (Pers.), *Auricularia auricula judae* (Bull. Ex St.Amans) Berck, *Marasmius oreades* (Bolt. Ex Fr.) Fr were purchased in season from the local market in Madrid (Spain). They were lyophilized and ground into fine powder as described by Ramirez-Anguiano *et al.* (2007)<sup>11</sup>. Dried mushroom powders were stored at -20 °C until further use.

Fresh *Lentinula edodes* fruiting bodies were also subjected to traditional cooking treatments (see later) or processed to separate the different tissues with a knife. Then, individual parts were treated as described above.

In order to test an eritadenine-enriched preparation as potential functional ingredient for the *in vivo* tests, an extract was obtained by stirring *L. edodes* powder with water (0.5 g/L) for 1 min at room temperature. The resulting suspension was centrifuged for 7 min at 7000 rpm at 10 °C and the obtained supernatant was immediately frozen and lyophilized. This fraction was an eritadenine-enriched extract containing 3.1 mg /g.

## 2.2. Reagents

Solvents such as methanol (HPLC grade) and acetonitrile (HPLC grade) were acquired from Lab-Scan (Gliwice, Poland). Ethanol (HPLC grade), diethyl ether (HPLC grade) and calcium chloride were purchased from Panreac (Barcelona, Spain). Hydrochloric acid (37%), trifluoroacetic acid (99%), sodium hydroxide, pepsin (from porcine gastric mucosa), sodium chloride, Trizma base, maleic acid, pancreatin (from porcine pancreas) and L- $\alpha$ -phosphatidyl choline (lecithin) were obtained from Sigma-Aldrich (Madrid, Spain) and D-eritadenine (90%) from SYNCHEM UG & Co. KG (Felsberg, Germany).

All additives used for culinary preparations were food grade: gelatin was acquired from Mondelez International (Madrid, Spain) and agar-agar (E406), sodium alginate (E401) and calcium lactate (E327) from Cuisine Innovation (Dijon, France).

## 2.3. Traditional culinary processing

*Lentinula edodes* fresh fruiting bodies were cut into slices and cooked (30 g) following four traditional methods: grilling, microwave cooking, frying and boiling. These treatments were carried out as described by Soler-Rivas *et al.* (2009)<sup>9</sup> in quadruplicate and the resulting cooked mushrooms were directly subjected to an *in vitro* digestion model or freeze-dried, ground and stored at -20°C until further analysis.

## 2.4. Modern culinary processing

Lyophilized shiitake fruiting bodies were subjected to modern culinary processes used on molecular gastronomy such as thickening (usually called 'texturization'), gelling and 'spherification'.

Powdered shiitake mushrooms (500 mg) were mixed with 50 mL water and 1 g agar-agar (as a thickener) and homogenized with a culinary blender (Minipimer 3MR320 Braun, Aschaffenburg, Germany). Afterwards, the mixture was heated up to 100 °C and maintained for 1 min. Then, an aliquot was collected with a 60 mL syringe and injected into a silicone tube (77 cm length and 0.5 cm diameter). The filled tube was partially folded, making loops, and submerged in an ice bath to cool down. The semi-solidified (texturized) gel obtained was extracted from the silicone tube by forcing air inside with another syringe. Then, the texturized shiitake powder resembled semi-transparent spaghetti with a mushroom taste.

Powder from the same shiitake batch (500 mg) was added to a previously hydrated gelatin solution obtained by dissolving a gelatin film (1 g) in cold water (50 mL) for 3 min. Then, the mixture was stirred at 75 °C for 2 min. Afterwards, homogenate was poured into a round mold and stored at 4 °C for 2 h allowing gel formation.

'Spherification' was carried out by mixing the shiitake powder (500 mg) and sodium alginate (400 mg) with 50 mL water and homogenizing them with a blender until a slightly viscose solution was obtained. The mixture was kept 5 min at room temperature to eliminate bubbles. Then, it was introduced into a 60 mL syringe and slowly dropped on a calcium lactate solution (44 mM). The surface of the liquid drops polymerized when placed in contact with  $\text{Ca}^{2+}$ , yielding jelly spheres. Spheres were left for approx. 3 min, transferred to a water bath to remove calcium excess, taken out with a perforated spoon and plated.

Each culinary process was carried out 4 times. The resulting preparations were directly subjected to an *in vitro* digestion model or freeze-dried, ground and stored at -20°C until further analysis.

## 2.5. *In vitro* digestion model

Fresh shiitake fruiting bodies, as well as the food preparations resulting from the traditional and modern culinary processes, were digested following the procedure described by Gil-Ramirez *et al.* (2014)<sup>1</sup> with modifications. Samples (15 g of traditional culinary processed shiitake or the modern preparations including 500 mg shiitake powder) were masticated by a volunteer for approx. 2 min and spat into a beaker. Milli-Q water was acidified with 6 M HCl (adjusting the pH to 2.0) and added (54 mL) to the masticated samples. The mixture was transferred to a thermostatic vessel at 37 °C with mild stirring and pepsin (275 mg) was also incorporated. Then, it was incubated for 1 h and stirred in a titrator device (Titrino plus, Metrohm, Herisau, Switzerland) simulating gastric digestion. Afterwards, intestinal digestion was initiated by adding 5 mM CaCl<sub>2</sub> and 150 mM NaCl and adjusting the pH to 6.0 by adding 0.5 M NaOH. Then, a pancreatic solution (6 mL) containing 20 mg pancreatin, 633 mg bile extract and 228 mg lecithin (in 50 mM Trizma-maleate buffer pH 7.5) was added and the pH was adjusted to 7.5 and maintained for 2 h using a viscotrode (Metrohm, Herisau, Switzerland) placed in the titrator device. The stirring level and temperature were the same than used in the gastric digestion simulation.

After the digestion process, digested samples were heated in a water bath at 80 °C for 10 min to inactivate digestive enzymes (eritadenine was resistant to this thermal treatment as indicated elsewhere<sup>12,13</sup>) and then subjected to centrifugation (7000 rpm 15 min) to separate the supernatant that was considered as the bioaccessible fraction.

Eritadenine bioaccessibility was estimated to be the ratio (%) between the amount of eritadenine in the bioaccessible fraction and that in the sample before the digestion process.

## 2.6. *Animals and diets*

Sprague Dawley adult (5 weeks old) male and female rats ( $n = 24$ ) were purchased from Charles River (San Cugat del Valles, Barcelona, Spain) and housed, separated by sex, in groups

of four animals per cage. Animals were maintained under controlled conditions of temperature, humidity and light ( $24 \pm 2$  °C, 40–60% humidity, 12 h:12 h light/ dark cycle) and had free access to water and food (commercial rodent maintenance diet A04; Scientific Animals Food & Engineering, Augy, France). After an adaptation period (6 days) animals were weighed and randomly divided into three groups per sex: control group (C) that remained with the standard diet, another group that was fed with a low eritadenine dose (10 mg per kg animal per day) (LE) and the third group was fed a higher dose (HE) (21 mg per kg animal per day). Diets were prepared mixing A04 chow with the corresponding amount of the eritadenine-enriched extract necessary to obtain the indicated eritadenine concentration per group. The nutrient composition of each diet is summarized in **Table 1**. Animals were maintained on this diet for 5 weeks with daily evaluation of behavioral (posture and activity) and physiological (fur and mucosa status, hydration and the presence of secretions and wounds) parameters were scaled by trained staff and weekly monitoring of weight gain. Feces were collected at the beginning and at the end of the experimental period and maintained at  $-20$  °C until further use.

The protocol was approved by the Institutional Animal Ethics Committee of La Paz University Hospital (Madrid, Spain) and procedures were performed in accordance with the EU Directive 2010/63/EU and the Spanish law RD 53/2013 regarding the protection of experimental animals.

#### *2.7. Biosafety and bioavailability studies*

Following the experimental feeding period (5 weeks), overnight-fasted rats were euthanized by intracardiac exsanguination under anesthesia with 1.5% isoflurane. Plasma was separated out by centrifugation (10 min at 5000 rpm) using sterile tubes pre-treated with EDTA. The supernatant collected was stored at  $-20$  °C until analysis. Plasma levels of total cholesterol (TC), HDL cholesterol, triglycerides, glucose and circulating renal and liver damage



biomarkers (creatinine, uric acid, bilirubin, alanine transaminase (ALT), aspartate transaminase (AST)) were measured in duplicate for each sample using a Covas C311 Autoanalyzer (Roche, Basel, Switzerland) specifically calibrated for rodent samples. All enzymatic colorimetric kits and internal quality controls were supplied by Roche (Basel, Switzerland).

Livers, spleen, kidneys and testis or ovaries were collected, weighed and washed in ice-cold PBS. Samples were processed for each tissue, keeping a fraction fixed in 10% neutral buffered formalin for 24 hours and the other part was immediately frozen in liquid nitrogen and storage at -80 °C until analysis. Fixed samples were embedded in paraffin for further analysis by immunohistochemistry. Afterwards, frozen liver samples were subjected to homogenization to detect eritadenine (see later).

## 2.8. Eritadenine determination by HPLC-DAD

Eritadenine was extracted from samples and quantified following two different methods. One of them (method 1) was based on the procedure of Enman *et al.* (2007)<sup>14</sup> with slight modifications. In this case, samples (1 g) were mixed with 20 mL of 80% methanol (v/v) and stirred in the dark for 3 h. Then, the mixture was filtered through a 14–18 µm pore size paper filter (GE Healthcare Europe GmbH 1240, Barcelona, Spain) and methanol was removed on a rotary vacuum evaporator (60 °C), keeping the sample protected from light. Afterwards, the dried extract was mixed with 10 mL Milli-Q water, washed 3 times with diethyl ether, mixed with 40 mL ethanol (4:1, v/v) and kept overnight at -20 °C. Finally, the sample was filtered through filter paper, and ethanol was removed using the rotary vacuum evaporator and water using a freeze-dryer. The resulting eritadenine extract was stored at -20 °C until further use.

The second extraction method (method 2) followed the Afrin *et al.* (2016)<sup>15</sup> procedure. Briefly, samples (1 g) were mixed with 10 mL of 60% ethanol (v/v) and stirred for 2 min. The mixture was subjected to centrifugation (7000 rpm, 15 min) in a Heraus Multifuge 3SR+ centrifuge (Thermo Fisher Scientific, Madrid, Spain) and the supernatant was carefully

collected. The pellet was extracted twice and supernatants were pooled together, filtered and dried as explained in method 1.

Eritadenine was extracted from livers by homogenizing the organs with an IKA Werke T8 Ultra Turrax (Ika Works Inc., Staufen, Germany) at maximum power. Resulting homogenates were mixed with 10 mL of 60% ethanol (v/v) and stirred for 2 min, following method 2.

The identification and quantification of eritadenine were carried out using an HPLC system (Pro-Star 330, Varian, Madrid, Spain) equipped with a PDA detector (Pro-Star 363 module, Varian, Madrid, Spain). Samples were dissolved in the mobile phase (5 mg/mL), injected (10  $\mu$ L) into a C18 Spherisorb ODS2 analytical column (4 x 250 mm, 5  $\mu$ m, Waters, Missisagua, Ontario, Canada) and developed at 0.5 mL/min with water : acetonitrile (98:2, 1% v/v TFA). Eritadenine was quantified at 260 nm using a commercial standard (0.004 – 0.25 mg/mL). The compound eluted after 10.5 min and showed the characteristic eritadenine UV-spectrum.

## 2.9. Statistical analysis

Differences were evaluated at 95 % confidence level ( $P \leq 0.05$ ) using one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison test. Statistical analysis was performed using SPSS V.13.0 software (SPSS Institute Inc., Cary, NC) and GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA).

## 3. Results and Discussion

### 3.1. Determination of eritadenine in several edible mushrooms

Eritadenine was firstly isolated from *Lentinula edodes*<sup>5</sup> but not many other species were investigated to study whether they might contain interesting concentrations. Therefore, before selecting shiitake as a source of the metabolite, a preliminary screening of a few other related mushroom species was carried out. Eritadenine was extracted from selected mushrooms following two already described methods because they both claimed to be specific

for eritadenine determination but their procedures were different.<sup>14,15</sup> Results indicated that indeed *L. edodes* was the mushroom with higher eritadenine concentrations but a few others species contained it too although in lower concentrations (Figure 1a). Extraction of eritadenine using method 1 yielded slightly lower levels of this metabolite than extraction using method 2 and this difference seemed to occur similarly within all the analyzed species. Apparently, the longer incubation times utilized in method 1 did not increase extraction yield.

*Lentinula edodes* contained 1.0 or 1.4 mg/g (depending on the method utilized), levels that were lower than those in some publications<sup>14,15</sup> but higher than others.<sup>16</sup> However, the fact that both methods indicated similar concentrations suggested that differences with respect to the other publications might be because of different cultivation conditions, processing, mushroom strains, etc. Other mushroom species such as *Marasmius oreades*, belonging to the same family as *L. edodes* (Marasmiaceae), showed 0.7 mg/g eritadenine (according to method 2), only half the shiitake levels, suggesting that closely related species might also be used to synthesize significant amounts of this compound. Eritadenine was also found in other mushrooms belonging to the same order as *L. edodes* (Agaricales) such as *Amanita caesarea* (0.6 mg/g), *Lyophyllum shimeji*, *Agaricus bisporus* and *Pleurotus ostreatus* (approx. 0.2 mg/g). But other mushrooms not so closely related, such as *Boletus edulis*, *Morchella conica* and *Lactarius deliciosus* also contained similar eritadenine concentrations (0.2 mg/g). Therefore, the presence of the compound in higher concentrations might be only attributed to the *Lentinula* genus.

### 3.2. Production of eritadenine by shiitake fruiting bodies

A more detailed study about the eritadenine biosynthesis was carried out by determining its production within different fruiting bodies tissues and during their development. The results indicated that differences between tissues were more pronounced when using method 2 than 1. Within the cap, eritadenine was present in higher concentrations in the epidermis

than in the dermis or gills (Figure 1b). The role of this compound in the mushroom metabolism has still not been elucidated although some studies suggested its involvement in defense (as antibiotic nucleoside) like many other derivatives from the secondary metabolism.<sup>17,18</sup> If this was the case, it seemed adequate to concentrate the compound in the tissue with direct contact with the environment. The stipe also contained the compound but in lower concentration being in concordance with previous observations indicated by Saito *et al.* (1975)<sup>16</sup> where lower levels of eritadenine were found in all the stipes compared to the caps of several shiitake strains.

Moreover, when eritadenine biosynthesis was studied during the development of the fruiting bodies, the results indicated that the metabolite was produced in similar concentrations during their complete growth since no differences were found within developmental stages (Table 2). Although mature mushrooms showed slightly higher eritadenine contents (using method 2) differences with earlier stages were not statistically significant. Therefore, since method 2 was an easier procedure and differences between samples were broader than method 1, method 2 was selected for further eritadenine quantifications.

### 3.3. Effect of traditional culinary treatments in eritadenine stability and bioaccessibility

In order to investigate whether eritadenine might be assimilated by shiitake consumers, the effect of traditional culinary treatments applied to the mushroom before its consumption was evaluated. Furthermore, processed fruiting bodies were submitted to an *in vitro* digestion model mimicking human digestion to determine eritadenine concentrations in the bioaccessible fraction.

The dry heat irradiated during grilling did not significantly affected eritadenine levels compared to raw mushrooms (Figure 2a). Apparently, eritadenine was heat stable as also indicated by previous studies.<sup>12,13</sup> However, when other cooking treatments involving water as

heat transmitter medium were used, such as microwave or boiling, eritadenine content was reduced. Temperatures in these processes were milder than grilling; therefore, its losses might be due to lixiviation into the aqueous medium because of its hydrophilic nature. But, when a lipid medium was utilized for frying, a decrease in eritadenine content was also noticed. Perhaps the deeper penetration of the oil through the mushroom tissues brought the higher reached temperature (160 °C) to more internal parts of the mushroom entering more in direct contact with the molecule. When dry heat is used, although the irradiated temperature is higher than frying (200 °C), only the skin and first outer layers of tissue receive such irradiation. The inner parts are cooked by their own water content (meaning maximum 100 °C) because of the cooling effect of water evaporation and because biological materials show very low thermic conductivities. The dry heat reaching the skin induced a Maillard crust that might impair the leaching out of constitutive water containing the eritadenine.

After the culinary treatments, shiitake mushrooms were treated with digestive enzymes at specific pH values that simulate mastication and stomach and intestinal digestion. The resulting water-soluble digestates were considered as the fraction that might reach the enterocyte layer in the intestine where absorption takes place. The results indicated that, although grilled mushrooms showed similar eritadenine values than raw mushrooms after the digestion, the compound from the grilled mushrooms was more bioaccessible since almost 69.3% of the compound was found in the bioaccessible fraction, while if raw mushrooms were digested only 26.9% was noticed (Figure 2a). Apparently, the heat treatment partially disintegrated the structural fibers and hyphae facilitating its release into the bioaccessible phase. It could be similar to the effect of the glucanases and chitinases utilized by Enman *et al.* (2007)<sup>14</sup> to extract higher eritadenine concentrations from shiitake tissues.

Moreover, if cooking and digestion are considered, the high temperatures reached during frying were not so detrimental because although part of the eritadenine was lost during the

process, it facilitated the release of the remaining levels into the bioaccessible fraction (59.1%) reaching levels higher than the other culinary treatments involving water (30.8% bioaccessibility after microwave cooking and 35.4% after boiling). The further degradation in the latter treatments could be due to the fact that once the eritadenine is leached in the medium after cooking, it might be more accessible to digestive enzymes. Nevertheless, the eritadenine levels that, in principle, still might reach the enterocytes are similar to those of raw mushrooms.

#### *3.4. Effect of modern culinary treatments in eritadenine stability and bioaccessibility*

The effect of novel culinary procedures such as thickening (called 'texturization' by chefs), spherification and gelatinization on the eritadenine concentrations was also tested. The eritadenine content of gelatinized mushroom powder was similar to the non-treated powder (raw), suggesting that the mild treatment required to prepare this gel did not negatively influenced its levels (Figure 2b). However, when the mushroom powder was subjected to texturization or spherification, a large reduction of eritadenine was noticed. During texturization high temperatures were used to dissolve the agar-agar but spherification was carried out at room temperature; therefore, degradation by heat could not be the reason for their lower eritadenine content. Moreover, the above results indicated that the compound resisted temperatures higher than 100 °C. Thus, the other possibility might be that, during the culinary procedure, eritadenine could be scavenged by the polysaccharides used to elaborate the dishes (alginate and agar-agar) forming complexes that hindered eritadenine release with the extraction method utilized (method 2). The fact that [Emman \*et al.\* \(2007\) \[12\]](#) suggested to use glucanases and chitinases for its extraction might also support this possibility, particularly because if the gel was generated using proteins such as gelatin, an easy release of the compound was noticed.

The eritadenine of the shiitake gels generated using proteins or agar-agar was protected from digestive enzymes as its levels remained the same after digestion (Figure 2b). However,

when spherification was carried out only 40.1% of the spherified eritadenine was bioaccessible; perhaps, the bonds between alginate and eritadenine were stronger than in the other two gels and were resistant to digestion. Nevertheless, its bioaccessible levels were similar to raw mushroom powder with no culinary treatment.

### *3.5. Biosafety of diets containing eritadenine*

The animal intervention was performed with male and female rats administrated two different eritadenine doses (high (HE) and low (LE)) and a control group (C) without the metabolite. The LE group received the same eritadenine concentration as previous animal studies since it was indicated as an effective dose with hypocholesterolemic effect in mice.<sup>7</sup> The HE group received double the amount to test not only its effectiveness but also its biosafety. However, the compound was administrated as a food-grade eritadenine-enriched extract to study its potential as a functional ingredient to formulate novel foods and determine whether the rest of the compounds included in the preparation interfered with or enhanced its bioavailability. During the whole intervention, the appearance, behavior and physiology of animals remained stable. Animal weight gain was recorded during the whole intervention and no differences were observed between groups (Figure 3). Independently of the studied sex, tissues collected and evaluated did not show damages or differences in weight compared with the control group (Figure 4). Based on those results, further immunohistochemical analyses were not performed. Plasma samples were determined to evaluate circulating lipid profile, glucose and biomarkers related to renal and liver function (Table 3). All circulating biomarkers determined were not different from those reported in the control group, except for AST concentrations (aspartate transaminase) that were significantly lower in the LE group than that in the C group (males and females) and total cholesterol that was significantly lower in the LE group than in the C group but only in males. But a slight increase of uric acid levels was also noticed with increasing extract administration.

### 3.6 Bioavailability and hypocholesterolemic properties of eritadenine

Eritadenine could be detected in livers using the previously described chromatographic method (Figure 5). Control rats did not show the eritadenine peak with R.T. 10.5 min while a compound with spectrum compatible with eritadenine could be detected in some of the livers treated with both doses. In 3 livers from the male rats treated with high eritadenine concentration, the molecule was present within 262.9 to 107.1  $\mu\text{g/g}$  fw while only 2 of those treated with lower dose showed eritadenine (41.9 and 101.3  $\mu\text{g/g}$ ). In the liver from female rats, concentrations were slightly lower, being detected only in 2 of the livers from rats administrated with the higher dose (77.4 and 89.6  $\mu\text{g/g}$ ) and only 1 of the livers administrated with the lower dose (67.5  $\mu\text{g/g}$ ).

Moreover, an approx. 24% lowering of the atherogenic index TC/HDL was noticed with the lower eritadenine concentration tested compared to the controls (Table 3). This reduction was slightly less pronounced than the approx. 29% noticed in mice for the same eritadenine concentration.<sup>7</sup> Differences could be due to the different animal/physiological conditions (mice were hypercholesterolemic) or because eritadenine was administered as a standard compound in the previous study. The latter case would indicate that the use of a fungal extract partially impaired the eritadenine bioavailability. Nevertheless, in the same study, *L. edodes* was also directly administered and effective lowering of cholesterol levels was reached (5 and 10% *L. edodes* administration induced, respectively, 10 and 39 % TC/HDL reduction). Similar values were obtained in this study for male rats (24 and 31 % reduction was noticed for the tested doses, administered as 4.8 and 10 % of the diet). Unfortunately, the previous study did not determine the precise eritadenine concentration in the mushroom, nor indicated the animal sex, so no further comparison could be made.

## 4. Conclusion



Besides *Lentinula edodes*, eritadenine was found in significant amounts in other mushrooms such as *Marasmius oreades* and *Amanita caesarea*. It was synthesized during the complete fruiting body growth, being present in higher concentrations in the skin. The molecule showed certain thermal stability since losses due to common culinary treatments were lower than 35% in all cases. Grilling was recommended more than other methods *e.g.* boiling or microwaving not only to maintain high eritadenine concentrations but because it also enhanced its bioaccessibility, probably by facilitating extraction of the compound from the food matrix or protecting it from digestive enzymes. Moreover, a careful selection of the food additives utilized in molecular gastronomy should be made since the use of hydrocolloids such as alginates or agar-agar might scavenge the compound impairing its bioaccessibility. Administration of eritadenine to rats, supplemented as a bioactive ingredient in a normal diet, did not induce damage to any organ or metabolic disorder when added at concentrations up to 21 mg per kg animal per day for 35 days; therefore, it could be considered as safe. It was detected in liver, suggesting that it was bioavailable and could reach that tissue. The extract reduced TC/HDL index in serum of male/female rats although it slightly increased uric acid levels. Therefore, functionalization of foods including an eritadenine-enriched extract obtained from shiitake mushrooms to design a hypocholesterolemic product could be possible since apparently it resists culinary processing, it was not toxic, and it was absorbed and was effective at lowering cholesterol in rats. However, clinical trials are still necessary to confirm these effects in humans.

### **Conflicts of interest**

There are no conflicts to declare.

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401   **Abbreviations**

402           SAHH (S-adenosyl-L-homocysteine hydrolase); PC (phosphatidyl choline); PE  
403 (phosphatidyl ethanolamine); LE (low eritadenine dose); HE (high eritadenine dose), ALT  
404 (alanine transaminase), AST (aspartate transaminase), LDL (low-density lipoprotein), HDL (high-  
405 density lipoprotein), TC (total cholesterol).

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**Table 1:** Nutritional composition of diets. Rodent standard diet (A04) supplemented with high and low doses of the eritadenine-enriched extract containing 3.1 mg/g eritadenine. Results are expressed as g/100 g in dry base.

	<i>High Dose</i>	<i>Low Dose</i>	<i>Control</i>
Carbohydrates	54	57	60
Protein	14.4	15.3	16
Lipids	2.7	2.9	3
Dietary fibre	3.6	3.8	4
Eritadenine-enriched extract	10	4.8	----

**Table 2:** Eritadenine content (mg/g dry weight) in different developmental stages of *Lentinula edodes* fruiting bodies. No statistical significant differences were found.

Developmental stage	Eritadenine content (mg/g)	
	Method 1	Method 2
Immature	1.23±0.01	1.46±0.03
Intermediate	1.01±0.09	1.44±0.13
Mature	1.23±0.01	1.71±0.02

**Table 3:** Plasma lipid profile, glucose concentrations and circulating liver and renal damage biomarkers (all values are expressed in mg/dL except for ALT and AST that are IU/L). Statistical significant differences were indicated with the symbol (\*).

	Males			Females		
	High dose	Low dose	Control	High dose	Low dose	Control
Total cholesterol (TC)	60.75±2.75	52.75±17.06 *	80.00±11.50	71.50±11.73	77.25±9.5	87.00±2.16
HDL-cholesterol	23.25±1.63	18.13±5.45	20.95±2.05	21.77±3.03	23.45±3.14	20.20±1.66
TC/HDL	2.62±0.09	2.89±0.10	3.80±0.25*	3.27±0.12	3.30±0.05	4.33±0.35*
Triglycerides	100.00±33.46	71.50±10.50	109.50±19.02	70.75±28.25	86.50±19.82	103.00±30.74
Glucose	105.50±14.15	85.25±3.30	78.50±16.38	84.25±9.54	101.00±14.49	87.00±9.31
Creatinine	0.29 ±0.01	0.33±0.06	0.33±0.06	0.31±0.02	0.31±0.03	0.35±0.04
Uric acid	53.75±9.61	41.75±2.22	43.00±5.72	51.25±6.19	47.00±4.55	43.25±3.59
Bilirubin	0.05±0.02	0.07±0.03	0.04±0.03	0.02±0.02	0.05±0.04	0.02±0.01
ALT	38.50 ±8.19	34.25±2.63	33.00±4.32	43.50±15.8	34.50±9.75	32.75±9.39
AST	98.25 ±9.74	95.00±12.68*	129.00±14.73	98.50±7.23	86.25±11.15*	119.75±16.88



**Figure captions:**

**Figure 1:** Eritadenine content (mg/g dry weight) in a) several mushroom species and in b) different fruiting body tissues from *Lentinula edodes* determined according to two different methods. Different letters (a-d) denote significant statistical differences between species for the same method ( $P \leq 0.05$ ).

**Figure 2:** Eritadenine content (mg/g dry weight) in *Lentinula edodes* submitted to different a) traditional and b) modern culinary treatments and after an *in vitro* digestion model. Different letters (a-b) denote significant statistical differences between culinary treatments and (A-B) between the same culinary treatment and after *in vitro* digestion ( $P \leq 0.05$ ).

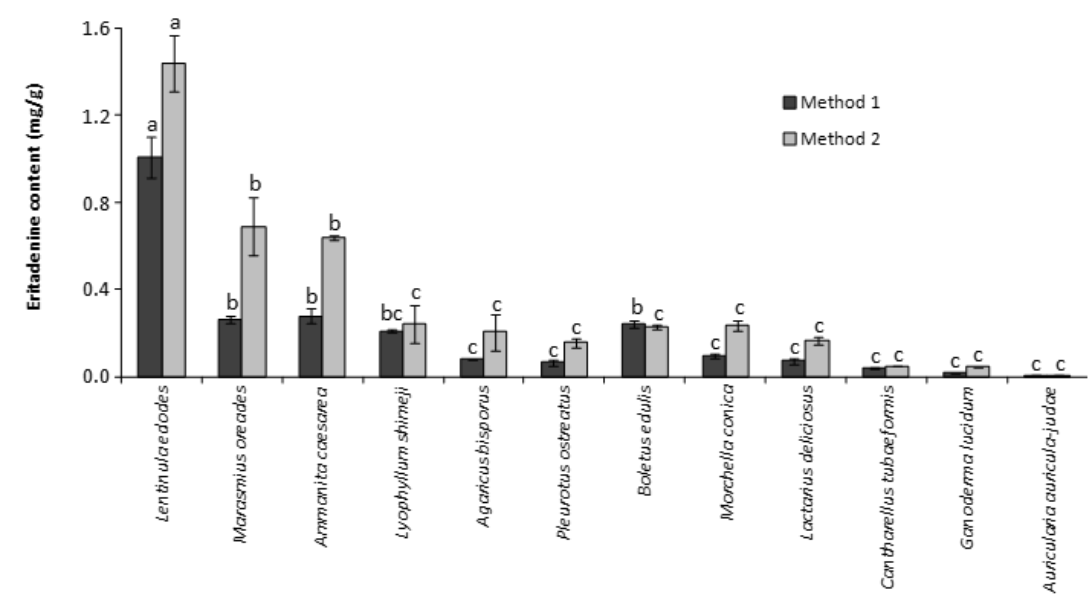
**Figure 3:** Body weight gain during eritadenine supplementation. a) Male and b) female animals supplemented with high dose (■) (0.021 mg/g animal /day) or low dose (◻) (0.01 mg/g animal /day) of eritadenine and control group (○). No statistical significant differences were observed between groups.

**Figure 4:** Organ weight after eritadenine supplementation. Total weight of spleen, liver, kidney and testis/ovary of a) male and b) female animals supplemented with high dose (black bars, 0.021 mg/g animal /day) or low dose (grey bars 0.01 mg/g animal /day) of eritadenine and control group (white bars). No statistical significant differences were observed between supplemented groups.

**Figure 5:** Chromatograms of liver homogenates obtained from rats supplemented with none (red line) or high (green line) eritadenine dose and an eritadenine standard (black line).

Figure 1:

a)



b)

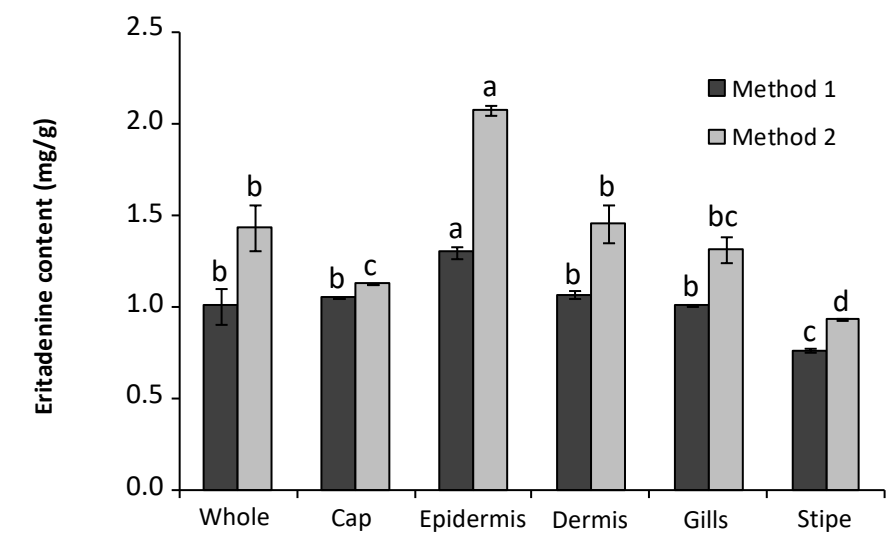
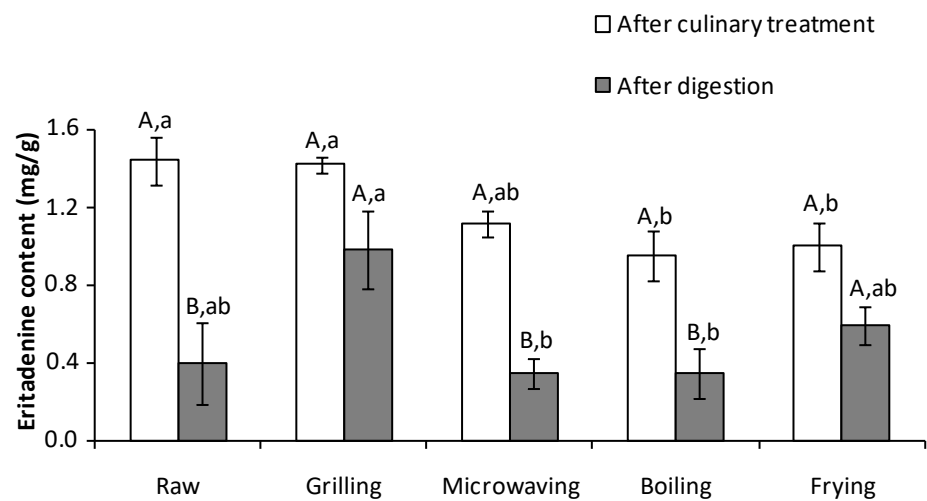


Figure 2:

a)



b)

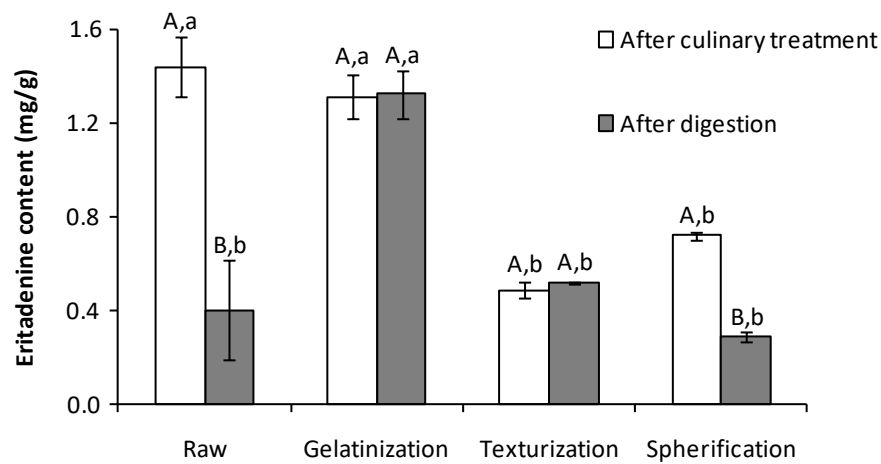
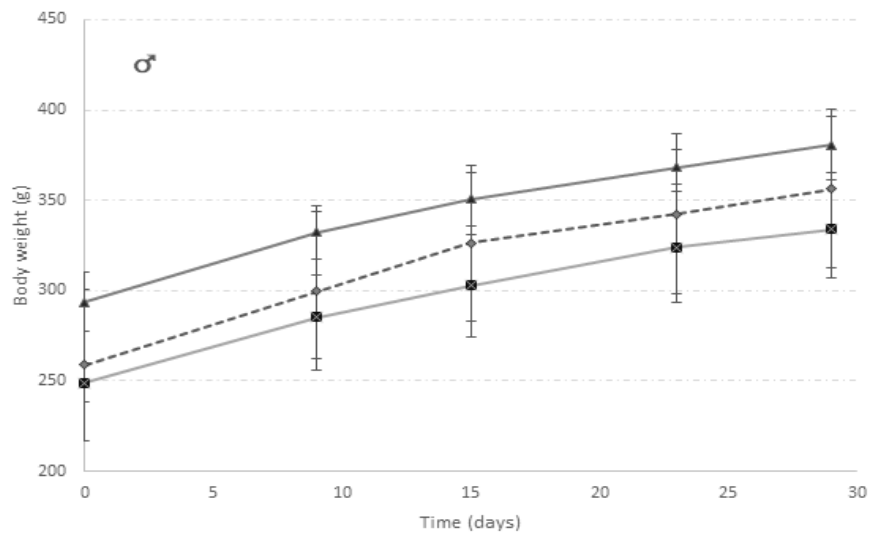


Figure 3:

a)



b)

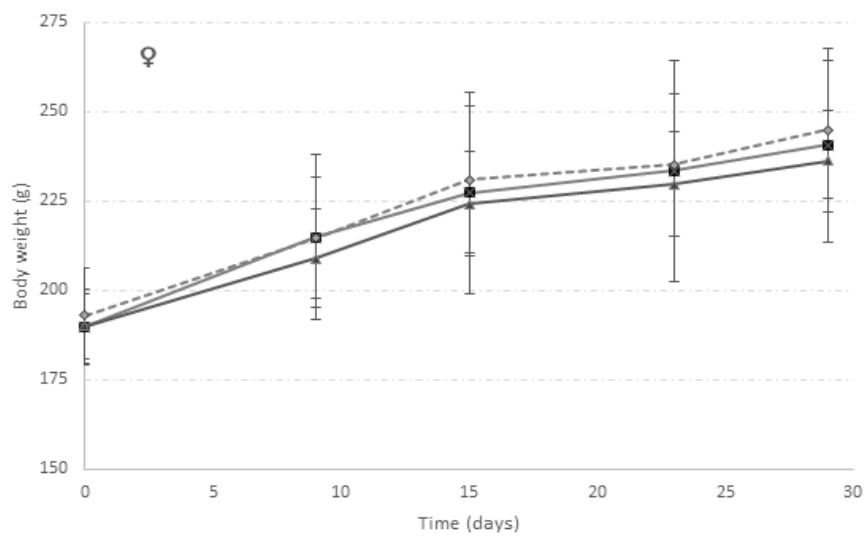
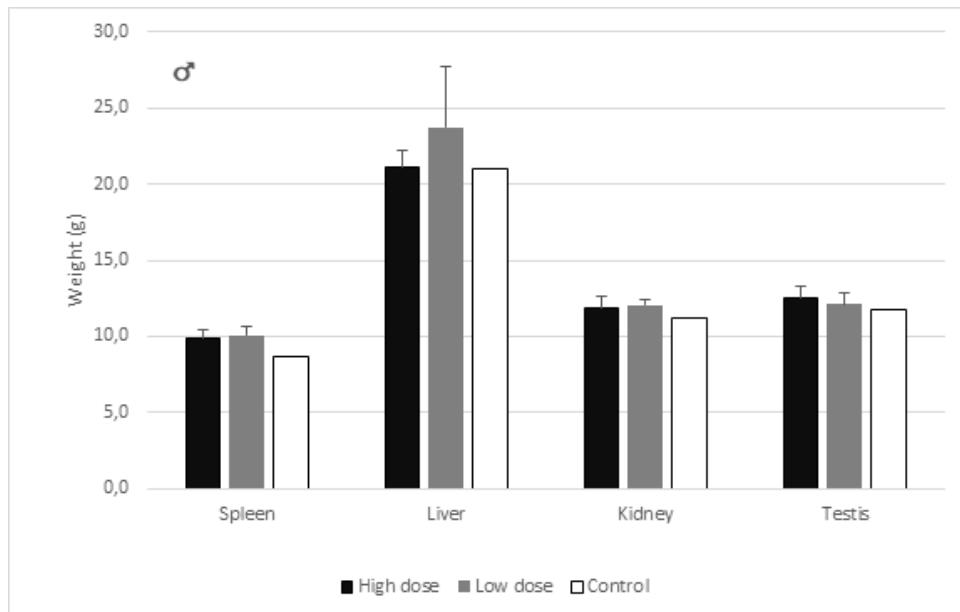


Figure 4:

a)



b)

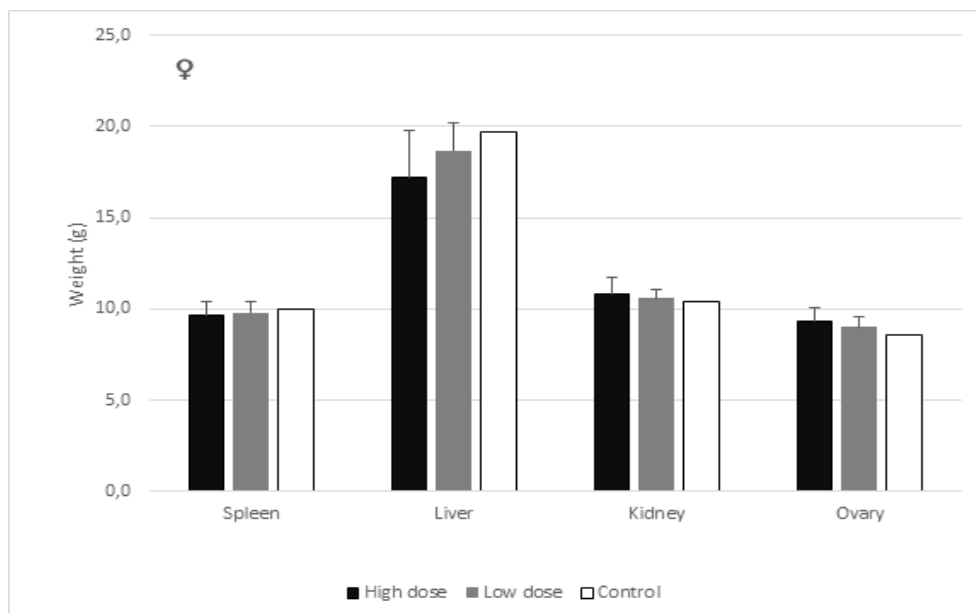


Figure 5:

