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Investigation of Lovastatin, the Anti-hypercholesterolemia Drug Molecule from Three Oyster Mushroom Species

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Abstract

Research on the availability of lovastatin in different mushroom species, mechanism of control of diseases like hypercholesterolemia and their drug efficacy are limited. Therefore, the present investigation is focused on quantitative and qualitative analysis of lovastatin from different highly preferred edible oyster mushrooms species by the consumers. The study was carried out for the determination of lovastatin content from different species of mushroom like, *Pleurotus ostreatus* (grey), *Hypsizygus ulmarius* (white) and *Agaricus djamor* (pink) under different culture conditions like Solid State Fermentation (SSF), Submerged Fermentation (SMF) and in the powder of dried mushroom of *P. ostreatus* as well. The lovastatin content was determined by UV spectrophotometric analysis and further separation, detection, quality and quantitative analysis were made in the partially purified lovastatin using High Pressure Liquid Chromatography. The lovastatin content was found to be highest in the *P. ostreatus* under all the given culture conditions.

Keywords

Pleurotus ostreatus (Grey), *Hypsizygus ulmarius* (White), *Agaricus djamor* (Pink), SSF, SMF, Lovastatin

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1. Introduction

Hypercholesterolemia also called dyslipidaemia is the presence of high levels of cholesterol in blood. It is a form of "hyperlipidaemia" an elevated level of lipid in the blood and "hyperlipoproteinamia" an elevated level of lipoproteins in the blood [1]. This can be sporadic which can occur with no family history or familial. Familial hypercholesterolemia is the most common inherited type of hyperlipidaemia. It predisposes to premature arteriosclerosis including coronary artery disease with heart attacks at an unusually young age. Hypercholesterolemia is typically due to a combination of environmental and genetic factors. Environmental factors include obesity and dietary choices. Genetic contributions are usually due to the additive effects of multiple genes, though occasionally may be due to a single gene defect such as in the case of familial hypercholesterolemia. A number of secondary causes exist including diabetes mellitus type 2, obesity, alcohol, monoclonal gammopathy, dialysis, nephrotic syndrome, hypothyroidism, Cushing's syndrome, anorexia nervosa, medications with thiazide diuretics, cyclosporine, glucocorticoids, beta blockers, retinoic acid [2].

Lowering cholesterol level reduces your risk of heart disease and stroke. Studies show that for every 1% reduction in cholesterol level there is a 2% reduction in the rate of heart disease. People who already have heart disease are at higher risk benefit most from lowering their cholesterol. Some of the herbs and medications have shown better performance in lowering the cholesterol. The herbs employed in treating the hypercholesterolemia are hawthorn, garlic, olive leaf extract,

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red yeast rice, psyllium [3] and guggul-garlic [4]. Medications like statins, niacin, bile acid sequestrates, cholesterol absorption inhibitors, fibric acid derivatives are also in use. Mushrooms are healthy source of food rich in proteins, vitamins, and minerals and relatively low in calories and fat. They constitute an increasing share in the world diet food contribution. Traditionally, medicinal properties of mushrooms have been well demonstrated particularly in eastern Asian countries. Currently, in some parts of the world, there is a renaissance of interest in traditional remedies. Mushroom lovastatin has high therapeutic value in combating hypertension, hypercholesterolemia, and cancer. Since, there are compounds like lectins, polysaccharides, polysaccharide-peptides, polysaccharide protein complex have been isolated from mushroom and these compounds have been found to have antioxidant, anticancer, antimicrobial, antidiabetic, anti hypercholestremic and immuno-modulatory properties.

The present study is aimed at investigation of lovastatin from edible mushroom species due to its few interaction with other drugs and easy intake. Lovastatin is a naturally occurring molecule found in food such as red yeast rice, oyster mushroom [5]. Lovastatin exhibit action beyond lipid-lowering activity in the prevention of hypercholesterolemia, Parkinson's, Alzheimer's disease and atherosclerosis. It also acts as anti-inflammatory agent and augments bone regeneration [6]. Lovastatin, also known as monocolin k, is an inhibitor of cholesterol biosynthesis, produced by *Pleurotus ostreatus* as a secondary metabolite [7]. It inhibits 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA), an important step in cholesterol biosynthesis [8]. Lovastatin is therapeutically used in free acids and lactone forms as well [9]. β-hydroxic acid forms of lovastatin are insoluble in water, whereas it is soluble in lactone ring form. Lovastatin not only reduces blood cholesterol [10] but also has an anti-fungal property and anti-carcinogenic effects [11], which is proved by inhibiting the growth of *Rhodotorula rubra* [12], *Mucor racemosus* [13]. The growth of *Candida albicans* also inhibits the β-hydroxic acid form of lovastatin.

In the present investigation, an attempt is made to isolate lovastatin molecules from three different types of oyster mushrooms under different culture conditions, characterized and tested its potential effect in lipid lowering ability under *in-vitro* conditions. The findings of the present investigation would yield the abundance and suitable source of elite mushroom species for lovastatin molecule for future drug development which would help to reveal the molecular mechanism of action of mushroom lovastatin on hypercholesterolemia and the drug dosage level of mushroom lovastatin for hypercholesterolemia in the novel strategy development including drug formulations.

2. Materials and Methods

2.1. Collection of Samples and Cytological Studies

Pleurotus ostreatus (Grey), *Hypsizygus ulmarius* (white) and *Agaricus djamor* (pink) were collected from National Horticulture Board (NHB), Bangalore, and Karnataka, India. The fungal cultures from spawn were maintained in Potato Dextrose Agar (PDA) media at 4°C. Morphological properties namely colony colour, shape, size, margins, elevation were observed and fungal culture mycelia were stained with lacto-phenol cotton blue for their microscopic properties [14]. Further inoculums were used for different cultivation, lovastatin production and extraction process.

2.2. Lovastatin Extraction, Estimation and HPLC Analysis

Extraction of lovastatin through SMF based on the methods described by Seenivasan et al., 2015 [15]. Cultures were inoculated to 100 ml production medium (g/L) containing glucose 50 g, yeast extract 20 g, tomato paste 30 g, oat meal 20 g, sodium acetate 10 g, ammonium sulfate 5 g, potassium sulfate 5 g, potassium dihydrogen phosphate 2 g, trace elements stock solution 10 mL at pH 7.0. After 10 days of inoculation, broth was acidified to pH 3.0 with 10% 1 N Hcl and content was extracted with equal volume of ethyl acetate under shaking condition (180 rpm) at 70°C for 2 hours. The fungal biomass was separated by filtration using pre-weighed Whatmann filter paper. Filtrate was centrifuged at 3000 rpm for 10 min and organic phase was collected. To the 1 mL of organic phase 1% glacial acetic acid was added for lactonization process. Then the extract was concentrated at 80°C, diluted to 1 mL with acetonitrile and filtered through filter paper.

Lovastatin from SSF media was made as per the methods described by Lakshmanan D and Radha K V, 2012 [16]. For solid state fermentation, the substrate rice straw was obtained from vendors nearby. The Rice straw was cut into 2-2.5 inches approximately, treated with tween- 20, washed under tap 3 to 4 times, then it was subjected to fungicide Bavistin (5%) and again washed under running tap water 3 to 4 times. The processed straw was autoclaved at 121°C under 15 lbs psi and air dried. 20 g of straw was then transferred into three 500ml conical flask, inoculated with three different varieties of Mushrooms spawns and incubated at 28°C for 8 days. Growth was regularly monitored and after the complete mycelial growth they were subjected to the lovastatin extraction. At the end of solid state fermentation (SSF), the fermented material was dried at 50°C for 24 h and powdered.

Lovastatin from fruiting bodies was extracted following the methods specified by Saswata Goswami et al., 2013 [17].

The ready to grow mushroom bags were obtained from NHB, Bangalore and the bags were kept into a sterile environment to avoid contamination. These mushrooms were harvested and dried at 60°C in hot air oven. From the dried mushrooms lovastatin was extracted with ethyl acetate by vortex agitation for 15 min and stored at cold condition for 1 h and centrifuged at 3000 rpm for 15min. Supernatant was collected for further analysis.

Lovastatin was extracted from the powdered (2g) material with ethyl acetate (pH 3.0) in 250 ml flasks and incubated at 28°C in rotary shaker at 100 rpm for 24 hrs. After 24 hrs, the mixture was centrifuged at 10,000 rpm for 10 min at room temperature and supernatant was filtered through filter paper. This supernatant was used for further chromatographic and spectroscopic analysis. The procedure was followed for all 3 varieties of mushroom.

The quantitative analysis of lovastatin was made as prescribed by Raghunath R et al., 2012 [18]. To 1 mL of the purified lovastastin extract, 1 mL of glacial acetic acid (1%) was added and incubated for 10 min for lactonization of hydroxyl acid form of lovastatin. From this solution, 0.5 mL was taken and diluted 10 times with methanol and its absorbance was read at 238 nm with methanol as blank by using UV-Visible Spectrophotometer (Elico, India) with Diode Array Detector.

All the samples obtained from SSF, SMF and mushroom fruit powder extracts were concentrated, dried, dissolved in acetonitrile and 20µl of the sample is subjected to the HPLC analysis (Waters, model- 510, India) to determine the presence and estimated the concentration of lovastatin against the commercial Lovastatin tablet (Lovastatin 10 mg, Dr. Reddy's) as standard at Azyme Bioscience Pvt. Ltd. Bangalore, India [16]. The tablet is powdered, dissolved in acetonitrile (10mg in 25ml mobile phase) and filtered to use for HPLC analysis. 20µl of the sample was injected in reverse phase C18 column (4.6 Dia mm, 250mm length). An isocratic condition was maintained in the mobile phase (acetonitrile and water 70:30V/V). The flow rate was 1.ml/min throughout the run, pressure 1300 psi and the detection was carried out at 238nm

3. Results and Discussion

The mushroom *P. ostreatus, H. ulmaris* and *A. djamor* spawns collected from NHB and they were cultured in PDA plates for their colony characteristics and stained with lactophenol cotton blue for mycelial characteristics and based on the colony, mycelia and spore characteristic features they are confirmed as *Pleurotus ostreatus* (Greyish white, elevated, cotton like appearance), *Hypsizygus ulmarius* (White, flat colonies, cotton like appearance) and *Agaricus djamor* (Pink flat colonies). Hall and Ian R (2010) [19], Christensen C M (1981) [20] and Paul Stamets (2000) [21] investigated *Pleurotus ostreatus*, *Hypsizygus ulmarius* and *Agaricus djamor* and observed similar characteristics on par with the findings in the present investigation. Lovastatin estimation was carried out for all the extracted samples before HPLC analysis and it was found that all the samples contain lovastatin and the differences in optical densities (O.D) were observed among the organism as well as in different extraction methods (Table 2). Highest concentration of lovastatin was found in the SSF and SMF methods of *Pleurotus ostreatus*, 0.413 and 0.401 respectively at 238 nm. The yield of extracted lovastatin is higher in *P. ostreatus* when compared to *H. ulmarius* and *A. djamor*.

Table 1. The colony characteristics of *Pleurotus ostreatus* (Grey), *Hypsizygus ulmarius* (white) and *Agaricus djamor* (pink) were inoculated in PDA media.

Organism	Colony Color	Characteristics
Pleurotus ostreatus	Grey	Greyish white, elevated, cotton like appearance, Spores appear smooth kidney shaped and the fruiting body appears to be oyster like gills with stalk.
Hypsizygus ulmarius	White	White, flat colonies, cotton like appearance, spores are cylindrical and appearance is similar to *P. ostreatus*.
Agaricus djamor	Pink	Pink, flat colonies, morphology of fruiting body is same as *P. ostreatus*, spores are cylindrical and smooth.

HPLC Based Qualitative and Quantitative Analysis of Lovastatin

Extracted lovastatin samples were separated and analysed in HPLC at 238 nm. The concentration of lovastatin in samples were compared against the commercial lovastatin tablets for the retention time and peak area. Lovastatin samples and standard showed similar peak area ranges and retention time which indicates the presence of lovastatin in the extracted samples. The lovastatin drug concentrations of different extracted samples were calculated (Table 2, Figure 1). The highest concentration of lovastatin was found in SMF, SSF and dried powder extract samples of fruiting bodies of *P. ostreatus* (grey) as 0.74mg, 0.30mg and 0.47mg respectively. Also, SMF and SSF *A. djamor* (pink) was seen high compared to *H. ulmarius* (white).

Spawns of all the three types were subjected to different cultivation methods such as SSF, SMF and the fruiting body of mushrooms are also dried and powdered before lovastatin extraction process. The OD at 238nm confirmed the presence of lovastatin and the quantity of lovastatin was determined in all the extracted sample. Raghunath et al. (2012) [18] have reported the quantitative analysis of Lovastatin and the results (OD) were 0.413, 0.401 in SSF and SMF extracts of *P. ostreatus*. They reported that the lovastatin content was

highest in *P. ostreatus*. HPLC analysis revealed the purity of the compound (quality) and the quantity among the three species and in their different extracts. Reshetnikov Sergey V G and Wasser Solomon P (2001) [22] determined amount of lovastatin produced from submerged culture of *P. ostreatus* (0.52 mg/ml) and Lakshmanan D and K V Radha (2012) [16] used rice straw as substrate for SSF of *P. ostreatus* and the concentration of lovastatin by HPLC was recorded as 0.041 mg/ml. The characterized lovastatin extracts will be then used in future studies, in cell assay for the evaluation of hypercholesterolemia on HeLa cell line. According to Raghunath et al. (2012) [18], the effect of Lovastatin on HeLa cell line was reported and the increase in concentration resulted better efficacy of lovastatin against the cancer cell lines. Lovastain has anticarcinogenic property and *P. ostreatus* is edible, easily cultivable and rich in lovastatin which would pave way for the development of new drug formulations or as additives in anticarcinogenic and antihypercholesterolemia drug formulations as well.

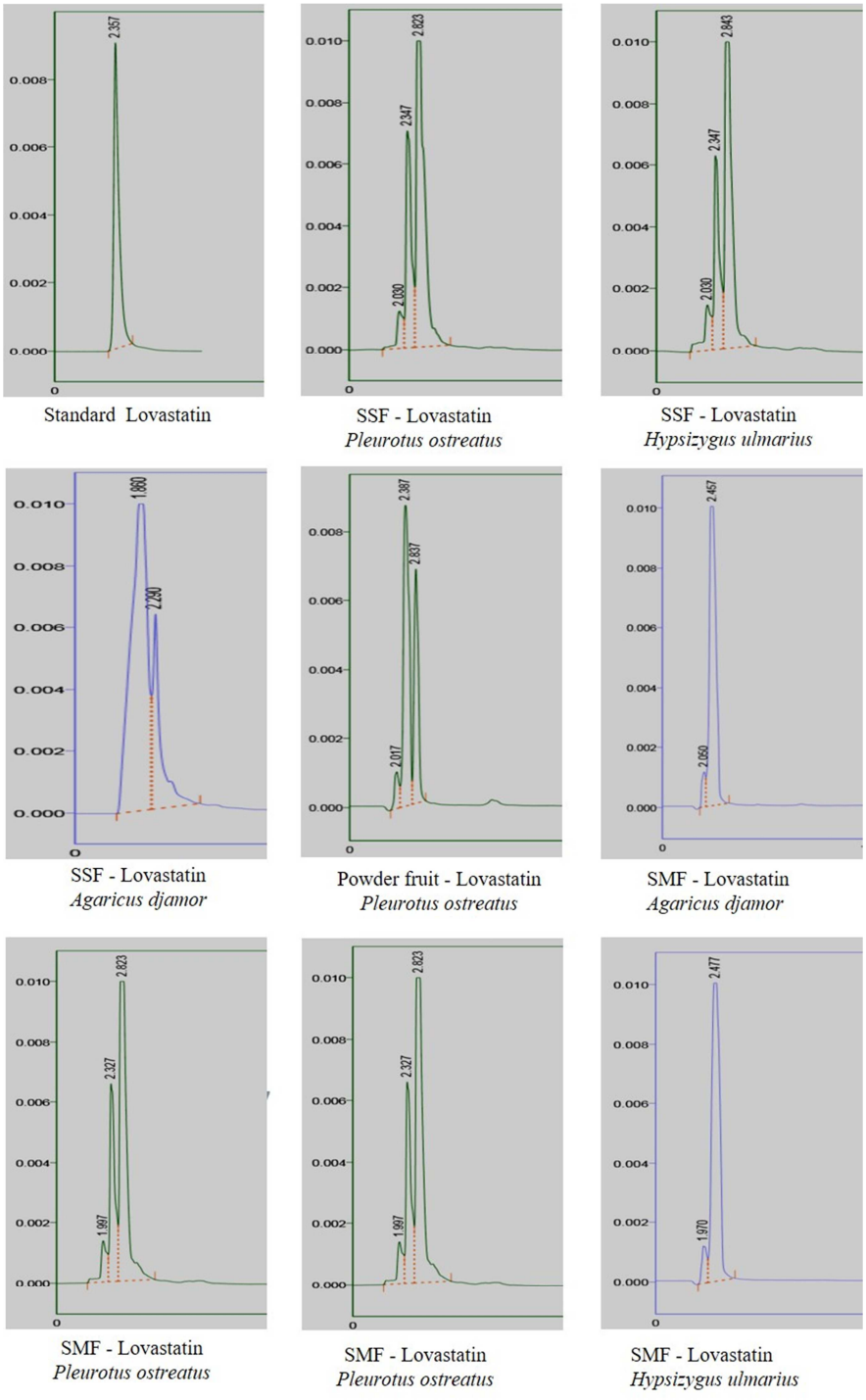

Figure 1. HPLC Chromatogram of Lovastatin in the standard and from three mushroom species extracted by different methods.

Table 2. Lovastatin level based on biochemical and HPLC analysis in three study fungus using different extraction methods.

Sl. No	Organism	Extract	OD at 238 nm	HPLC analysis		
				RT (min)	Peak area (mV.s)	Lovastatin (mg/ml)
1	*Pleurotus ostreatus*		0.413	2.3	362.1	0.33
2	*Hypsizygus ulmarius*	SSF	0.322	2.3	322.8	0.28
3	*Agaricus djamor*		0.315	2.2	429.4	0.30
4	*Pleurotus ostreatus*		0.401	2.4	338.2	0.74
5	*Hypsizygus ulmarius*	SMF	0.336	2.4	252.0	0.31
6	*Agaricus djamor*		0.343	2.4	247.0	0.70
7	*Pleurotus ostreatus*	Powdered fruit body	0.359	2.3	253.2	0.47
8	Lovastatin	Standard	-	2.3	126.5	0.20

4. Conclusions

Spectrophotometry and HPLC based quantitative, qualitative analysis of different extracts from three edible mushroom species inferred the lovastatin presence, purity and its concentration. Lovastatin was highest in *P. ostreatus* under solid state fermentation as well as submerged fermentation. *P. ostreatus* (grey) would be one of the appropriate sources for drug lovastatin which acts on cholesterol by inhibiting the HMG CoA Reductase enzyme. HMG CoA is involved in the synthesis of cholesterol and the lovastatin inhibits the formation of cholesterol from precursor mevalonate. Anti-hypercholesterolemia property of this drug has impact on hypertension, myocardial infarction and other heart diseases. Further studies on the lovastatin extracts to employ the cell assay to observe the anti- hypercholestremic effect using HeLa cell lines would determine the drug dosage level of mushroom lovastatin for hypercholesterolemia under in-vitro cell culture condition.

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