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Production of Secondary Metabolite (Lovastatin) From Edible Mushrooms *Agaricus bisporus* and *Calocybe indica*

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Abstract

Aim: In the present study an attempt was made to isolate Lovastatin from two different species of mushrooms such as *Agaricus bisporus* (white button mushroom) and *Calocybe indica* (white milky mushroom) and to estimate its potential toxic effect against HeLa by MTT assay. **Methods:** The Fruiting body of *Agaricus bisporus* and *Calocybe indica* were dried, powdered and extracted with ethyl acetate at 28 ˚C for 24 hours and estimated for the quantitative analysis of lovastatin through the UV-spectrophotometer at different nanometer from 230-260. The isolated lovastatin was subjected to HPLC analysis for the confirmation. **Results:** The maximum absorbance was obtained at 240 nm for *Agaricus bisporus* with the absorbance OD value 3.4618 and also at 240 nm for *Calocybe indica* with the absorbance OD value 3.509. Lovastatin samples showed similar peak area ranges and retention time was 2.148 for *A.bisporus* and for *C. indica* the retention time was at 2.527 which indicates the presence of lovastatin in the extracted samples. Cytotoxicity is one of the properties of anticancer agents. The effective concentration of 50% cell death was found to be at 31.2µg/ml or the extracts of *A.bisporus* and 62.5µg/ml *C. indica* respectively on human cervical cancer cell lines (HeLa cell lines). **Conclusion:** The presence of lovastatin a cholesterol lowering agent has been confirmed in *A.bisporus* and *C. indica* spectroscopic and chromatographic analysis. Hence these mushrooms can be used as a functional food with cholesterol lowering effects.

Keywords
Lovastatin, *Agaricus bisporus, Calocybe indica,* He La cells.

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INTRODUCTION

Cholesterol is a fat-like substance which is present naturally in the human body which plays a major role in body metabolism and membrane transport [1]. (Lakshmanan *et al.,* 2013) But high of cholesterol may lead to coronary heart disease. The body uses cholesterol as the starting point to make estrogen, testosterone, vitamin D, and other vital compounds. Cholesterol is categorized into two types i.e. low-density lipoprotein and high-density lipoprotein. Low

density lipoprotein can be controlled by maintaining normal diet and exercise. People who had LDL levels of 160 or higher are 70% to 90% more likely than those with LDL cholesterol levels below 100 to die from cardiovascular disease.

Elevated blood concentrations of total cholesterol or LDL cholesterol (LDL-C) increase the risk of cardiovascular disease (CVD), whereas higher concentrations of high density lipoprotein cholesterol (HDL-C) decreases [2] (Alarcon *et al.,* 2005). Studies have shown that dietary patterns high in saturated fatty acids, cholesterol, and animal fat increase low-density lipoprotein (LDL) cholesterol levels.

Statins are very effective in lowering the low density lipoprotein and hence reduce cardiovascular diseases. Lovastatin is a secondary metabolite produced from various fungi, which inhibits 3-hydoxy 3-methyl glut aryl co -enzyme (HMG-COA) which acts as an inhibitor enzyme for the cholesterol bio-synthesis in human and animals. Of many statin molecules, lovastatin and mevastatin are natural statins The Food and drug administration (FDA) approved lovastatin in 1987 for the treatment of hypercholestermia. Lovastatin reduces blood cholesterol level and it has also been reported to have anti-fungal and anti-carcinogenic effects. The nutritional and medicinal values of mushrooms have long been recognized. Mushrooms are found to be rich sources of protein, lipids, amino acids, glycogen, vitamins and mineral elements. *Calocybe indica* is commonly called as milky mushroom. Milky mushroom is milky white, spongy, umbrella like with resembles of button mushroom. Because of its large seized sporphores, simple production and low capital investment it is most favorable among marketers It is most abundantly grow in south Indian states. *Agaricus bisporus* is commonly called as white button mushroom.

In the present study an attempt was made to isolate Lovastatin from two different species of mushrooms such as *Agaricus bisporus* (white button mushroom) and *Calocybe indica* (white milky mushroom) and to estimate its potential toxic effect against human cervical cancer cells (HeLa) using MTT assay.

METHODOLOGY

Collection of samples

Commercially available fruiting bodies of mushrooms *Agaricus bisporus* and *Calocybe indica* were used.

Extraction process

200 gm of the whole sporocarp (inclusive of stipes, middle part and region attached to the pileus) were dehydrated by air drying at room temperature at 28℃ for12 hours and was ground into fine powder. The dried mushroom powders were stored at 20℃ until further use. Lovastatin was extracted from powdered material (5gm) with 50 ml ethyl acetate (pH 3.0) in a 250 ml conical flask and incubated at 28° C for 24 hours. Then, the mixture was filtered through filter paper and supernatant was collected and used further techniques [1].

Qualitative analysis of lovastatin

The qualitative analysis of lovastatin was made as specified by Raghunath *et al* 2012. To the 0.5 ml of lovastatin extract, 0.5 ml of glacial acetic acid (1%) was added and incubated for 10 minutes for the hydroxyl form of lovastatin. From this solution 0.5ml was taken and diluted 5 times with methanol and then absorbance was read at wavelengths ranging from 230-260 nm with methanol as blank by UV-spectrophotometer [3].

HPLC analysis of extracts of *Agaricus bisporus* and *Calocybe indica*

20µl of the clear extract was injected in shimazudu liquid chromatography [1].

Anticancer activity of the extracts on He La cell lines

HeLa cell line was obtained from NCCS, Pune. The cells were maintained in Minimal Essential Medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO2 at 37 °C. In vitro anticancer activity was estimated by MTT assay on He La cell lines [4]. Cells were plated in 24-well plates and incubated in 37℃ with 5% CO2 condition. After the cell reaches the confluence, the various concentrations of the samples were added and incubated for 24hrs. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or DMEM without serum. 100 µl/well (5 mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells. The absorbance at 570nm was measured with UV- Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC50) was determined graphically. The % cell viability was calculated using the following formula:

% cell viability = A570 of treated cells / A570 of control cells × 100

RESULTS

Extraction of lovastatin

200gm of mushroom fruiting body yielded 7 gm of fine powder. The powders were extracted with ethyl acetate to obtain 1.5-ml of crude extract.

Qualitative analysis of lovastatin

The presence of lovastatin was studied spectrophotometrically from the extracted samples of *Agaricus bisporus* and *Calocybe indica.* Absorbance was recorded from a range of 230 to 350 nm. (Graph 1, 2)

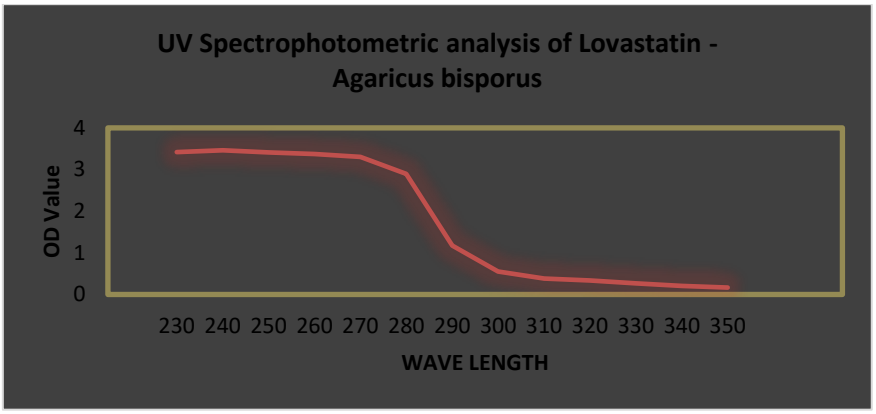
HPLC analysis of extracts

To confirm the production of lovastatin the extracts of *A. bisporus* and *C. indica* were subjected to HPLC analysis. The concentration of lovastatin in samples were compared against the literature references for the retention time and peak area. Lovastatin samples showed similar peak area ranges and retention time as reported earlier which indicates the presence of lovastatin in the extracted samples. The lovastatin drug concentrations of different extracted samples were calculated (Table 1, 2). Chromatogram analysis of extract of *A.bisporus* indicated the peak of lovastatin with the retention time of 2.527 minutes Fig 2(a). Chromatogram analysis of extract of C. *indica* indicated the peak of lovastatin with the retention time of 2.148 minutes Fig 2(b). The retention time were much similar to the retention time obtained in references.

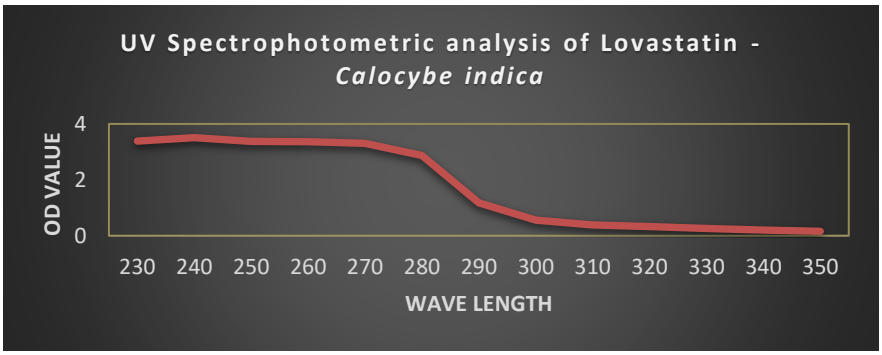
Anticancer activity of the extracts on He La cell lines:

The extract of *A.bisporus* could inhibit the proliferation of He La cells till a concentration of 31.25 µg/ml. The concentration of extract at 1000 µg/ml, 500 µg/ml 250 µg/ml, 125 µg/ml, 62.5 µg/ml and 31.25 µg/ml showed anticancer activity of 14.35%, 20.95%, 28.05%, 35.23%, 42.49% and 49.67% (Table 3) respectively that was comparable to that of positive control. The IC50 value of extract of A.bisporus after 24 hours was found to be 49.67% at 31.25µg/ml concentration.

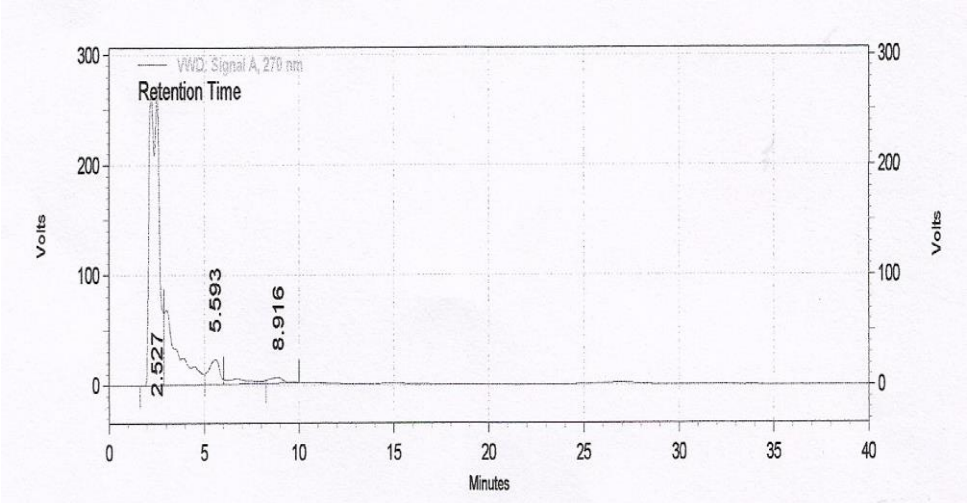
The extract of *C. indica* could inhibit the proliferation of He La cells till a concentration of 62.5µg/ml. The concentration of extract at 1000µg/ml, 500µg/ml 250µg/ml, 125µg/ml and 62.5 µg/ml showed anticancer activity of 18.90%, 26.8%, 34.65%, 40.43% and 48.27% (Table 4) respectively in comparison to control. The IC50 value of extract of Calocybe indica after 24 hours was found to be 48.27% at 62.5µg/ml concentration. Both the mushroom extracts showed a potent anticancer activity against the HeLa cells. The effect of the extracts was recorded as micrographs.



Graph 1: Graphical representation for qualitative analysis of lovastatin by *Agaricus bisporus*



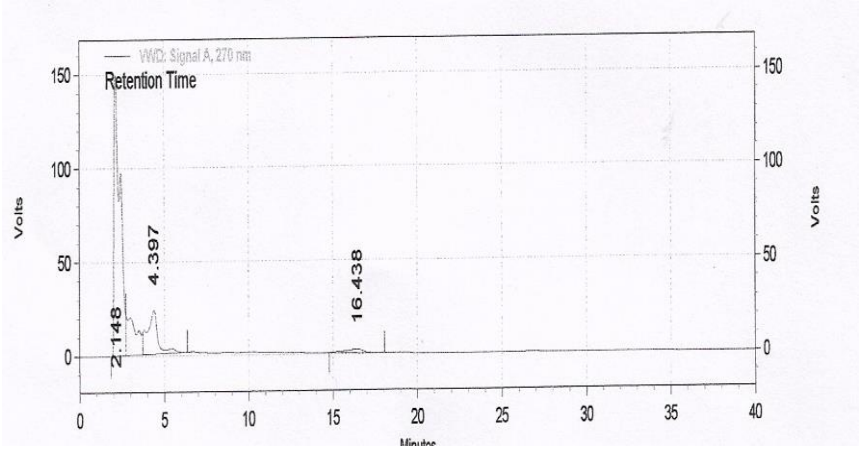
Graph 2: **Graphical representation for qualitative analysis of lovastatin by *Calocybe indica***



Graph 2 (a): The chromatogram showing retention time in *Agaricus bisporus*

S.NO	RETENTION TIME	AREA	AREA%	HEIGHT	HEIGHT%
1.	2.527	156045172	89.04	4562595	90.88
2.	5.593	15018625	8.57	377063	7.51
3.	8.916	4188702	2.39	80788	1.61
TOTAL	____	175252499	100.00	5020446	100.00

Table 1: HPLC analysis showing values at different retention time for *Agaricus bisporus*



Graph 2 (b): The chromatogram showing retention time in *Calocybe indica*

S.NO	RETENTION TIME	AREA	AREA%	HEIGHT	HEIGHT%
1.	2.148	6.1810294	75.98	2507307	85.53
2.	4.397	17149137	21.08	391171	13.34
3.	16.438	2395700	2.94	33105	1.13
TOTAL	____	81355131	100.00	2931583	100.00

Table 2: HPLC analysis showing values at different retention time for *Calocybe indica*

S.NO	CONCENTRATION (µg/ml)	DILUTION	ABSORBANCE (O.D)	CELL VIALBILTY (%)
1	1000	Neat	0.196	14.35
2	500	1:1	0.286	20.95
3	250	1:2	0.383	28.05
4	125	1:4	0.481	35.23

5	62.5	1:8	0.580	42.49
6	31.2	1:16	0.678	49.67
7	15.6	1:32	0.771	56.48
8	7.8	1:64	0.864	63.29
9	Cell control	-	1.365	100

Table 3: Anticancer effect of extracts of *A. bisporus* on HeLa cell line

S.NO	CONCENTRATION (µg/ml)	DILUTION	ABSORBANCE (O.D)	CELL VIALBILTY (%)
1	1000	Neat	0.258	18.90
2	500	1:1	0.367	26.88
3	250	1:2	0.473	34.65
4	125	1:4	0.552	40.43
5	62.5	1:8	0.659	48.27
6	31.2	1:16	0.764	55.97
7	15.6	1:32	0.852	62.41
8	7.8	1:64	0.942	69.01
9	Cell control	-	1.365	100

Table 4: Anticancer effect of extracts of *C. indica* on HeLa cell line

DISCUSSION

The EFSA (European Food Safety Authority) has recognised two types of functional foods as able to reduce cardiovascular diseases, the foods containing phytosterols and β glucans. Edible mushrooms are good sources of both. Also, there are previous reports showing the presence of lovastatin in few edible oyster mushrooms, a compound which is able to lower cholesterol levels by inhibiting HMG CoA reductase a key enzyme in cholesterol metabolism. Hence the current study was aimed to preliminary screen for the presence of this potentially active compound lovastatin from *A. bisporus* and *C. indica*

There are several reports available for lovastatin biosynthesis using different organisms and *Pleurotus osteratus* mushroom varieties. However, there has been no study reported so far on the use of *Agaricus bisporus* and *Calocybe indica* for extraction of lovastatin. At the beginning of mushroom growth lovastatin is distributed uniformly in the entire small sporocarp and there is no difference in the level of lovastatin in the pileus and the stipe. But as the sporocarp grows the majority of the lovastatin is transfer red to the pileus [5], hence extracts were prepared form the powder obtained from whole sporocarp of commercially obtained mushrooms.

Lovastatin exists as lactone form and hydroxyl acid form. The absorption maxima for both the forms of lovastatin appears similar. The current study identified lovastatin based on the absorbance of the spectrophotometer and graphs were obtained for *A. bisporus* and *C. indica* at different concentrations (Graphs 1 and 2). Various absorptions were taken over the range of 230 to 350 nm. The maximum absorbance was obtained at 240nm for both the extracts of *A. bisporus* and *C. indica*. The extracts had a very mild variation in absorbance. The results were in agreement with previous studied literature which represented diversity [6], using *Pleurotus osteratus* for production and reported maximum absorbance peak for β- hydroxyl form of lovastatin at 220nm and in study on *Monascus purpureus* reported pure lovastatin to have three absorption maxima at 232, 238 and 247 nm in UV- visible spectrophotometer [7]. This proves that the extracts obtained from *A. bisporus* and *C. indica* had lovastatin pure and in conformity to other literatures. Lovastatin was observed in both the samples and the differences in optical densities were observed among organisms as well as in extraction methods.

Analysis of lovastatin in *A. bisporus* and *C. indica* was carried by HPLC method. The extracts were eluted at a retention time of 2.148 minute for *C. indica* and 2.527 minute for *A. bisporus* which were much similar to the retention time obtained by earlier studies [8] in *Pleurotus osteratus* and *Agaricus djamor.* The elution obtained at 2.148 and 2.527 confirms lovastatin. Cytotoxicity is one of the properties of anticancer agents. The effective concentration of 50% cell death was found to be at 31.2µg/ml or the extracts of *A. bisporus* and 62.5µg/ml *C. indica* respectively on human cervical cancer cell lines (He La cell lines). It has been suggested that statins reduce cancer incidence by 28 – 33% [3]. These results demonstrate lovastatin to have strong anticancer effect.

CONCLUSION

The present study concludes that lovastatin can be obtained from *Agaricus bisporus* and *Calocybe indica*. So far lovastatin production has been reported in *Pleurotus ostreatus* mushroom. This investigation has documented the isolation and production of lovastatin from *Agaricus bisporus*, the button mushrooms and *Calocybe indica*, milky mushrooms. The study confirmed through spectroscopic and chromatographic analysis the presence of lovastatin. Hence the fruiting bodies or the extracts of the *Agaricus bisporus,* button mushrooms *Calocybe indica*, milky mushroom might be considered as a source of cholesterol lowering compound lovastatin.

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