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Advances in Bioresearch

ORIGINAL ARTICLE

Isolation and Screening of Lovastatin from Hybrid Mushroom

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ABSTRACT
Lovastatin is commercially available from various entophytic microbes, where, the high yield of lovastatin production is identified from fungal species; it can be isolated from secondary metabolites like red yeast and some filamentous fungus. This research article entitles about the isolation and screening of lovastatin production in hybrid which is identified as mushroom produced by two different species, in such consideration the hybrid species is cultured from red yeast Monascus purpureus. and filamentous fungi like Pleurotus ostreatus. Thus this paper focuses about the isolation, screening and analysis of lovastatin level in the obtained hybrid mushroom.
Keywords: Monascus purpureus, Pleurotus ostreatus, Lovastatin, Antioxidant, Hybrid, TLC , HPTLC , IR.

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INTRODUCTION

Lovastatin can be obtained from different sources like mushrooms, dark fermented tea and endophytes. Commercially, lovastatin is produced from endophytic microbes. Till date the highest yield of lovastatin has been reported from endophytic fungal species, *Aspergillus terreus*[1]Lovastatin was first isolated as a secondary metabolite from a red yeast *Monascus ruber* and a filamentous fungus *Aspergillus terreus*. Then, several fungal genera including *Aspergillus* spp., *Trichoderma*, and *Pleurotus etc.*, are also reported as best lovastatin promotor [2]. . Even though *Aspergillus sp*. especially *A. terreus* has been reported to be the highest lovastatin producer with 180 mg lovastatin/L18, further investigations have to be conducted on screening of high yielding strains MTCC 369 and 1801of *Monascus .purpureus* and *Pleurotus ostreatus* under-utilized sources. Where, this article mainly focus about the screening and analysis of lovastatin level in the hybrid mushroom, from the isolated hybrid the potential of lovastatin was screened for its highest activity in a flask system.

Hypercholesterolemia is a potent risk present in the world due to modern lifestyle. It is a silent killer which will claim nearly 3.5 million lives in upcoming decade according to WHO. Lovastatin also known as 'Merck's Mevacor' is an anti-cholesterol agent. Lovastatin blocks the cholesterol synthesis by acting as a competitive inhibitor of HMG – CoA reductase and thus inhibits the mevalonate pathway. However, due to limited availability of lovastatin, there is an utmost need to explore alternative natural sources to meet its scarcity. Several fungal genera including *Aspergillus terreus, Penicillium citrinum, Monascus ruber and Pleurotus spp*. have been reported to produce lovastatin.

MATERIAL AND METHODS

Taxonomical Investigation of selected (PO3) Hybrid strain

The isolated hybrid strain was grown for 7 days on different growth culture media: (PDA,SDA,RBA).PDA (potato dextrose agar) containing (g/l) : Potato infusion from-200.00g/l, Dextrose -20.00g/l, Agar-30.00g/l, Final pH at (25°C)5.6 ± 0.2. SDA (Sabouraud Dextrose Agar) containing(g/l) :Mycological peptone -10.00g/l, Dextrose-40.00 g/l, Agar 15.00 g/l, Final pH at (25°C) 5.6 ± 0.2. RBA (Rose Bengal Agar)Dextrose-10.00 g/l, Magnesium sulfate -0.5g/l, chloramphenicol- 0.1g/l, Rose Bengal-0.05g/l,Agar-15.0g/l

All culture media were sterilized at 121°C. The cultivation was performed in petri dishes at 25°C for 7 days for all culture media. Additionally cultivation with pH5 at 25°C was carried out. During the cultivation, morphological and cultural characteristics of hybrid was observed using Lacto Phenol Cotton Blue (LPCB) staining technique.[3]

Screening of lovastatin in hybrid

Lovastatin can be obtained from different sources like mushrooms, dark fermented tea and endophytes. Commercially, lovastatin is produced from endophytic microbes. Till date the highest yield of lovastatin has been reported from endophytic fungal species, *Aspergillus terreus* [1]

Lovastatin was first isolated as a secondary metabolite from a red yeast *Monascus ruber* and a filamentous fungus *Aspergillus terreus*. Then, several fungal genera including *Aspergillussp*., *Penicillium*, *Monascus*, *Paecilomyces*, *Scopolariopsis, Doratomyces, Phoma, Phythium, Gymnoascus, Trichoderma*, *Hypomyces* and *Pleurotus*, are also reported as lovastatin producers. Even though *Aspergillus sp*. especially *A. terreus* has been reported to be the highest lovastatin producer with 180 mg lovastatin/L18, further investigations have to be conducted on screening of high yielding strains MTCC 369 and 1801 PO3(hybrid) of *Monascus purpureus* and *Pleurotus ostreatus* under-utilized sources. Where, this chapter mainly focus about the screening and analysis of lovastatin level in the hybrid oyster mushroom, from the isolated hybrid the potential of lovastatin was screened for its highest activity in a flask system.

Isolation of lovastatin from hybrid

The isolation technique primarily involves sub culturing and maintains of pure culture, the procurement plates of hybrid were maintained by MTWU Kodaikanal, India. The hybrid culture on PDA plates was preserved for long term for standard results on mutual studies. A standard lovastatin producing fungal strain GG3F16 was provided, the isolates from hybrid was added into 10ml of sterile distilled H_2o, until final dilution of 10-9 which was applied on to PDA plates using spreading technique, where the plates were incubated at room temperature for a week, colony were formed, later the pure single colony was transferred onto PDA plates and slant.

Production of lovastatin by SSF

Solid substrate fermentation (SSF) was done to examine the production of lovastatin, where a combination ratio of 1:1 5g rice bran and brown rice was placed in 250mL Erlenmeyer flask. The moisture content was adjusted to pH 6.5 by adding distilled H_20.the flask were sealed with cotton plug and foil and autoclaved at 121°c for 15 min. After cooling the broth, it was inoculated with 1mL spore solution with a size of 1 x 107 (spores/mL) it was mixed thoroughly to have a uniform spore distribution in the substrate, the inoculated media were incubated at room temperature for a week aerobically[4].

Screening of lovastatin production

The initial stage of lovastatin presence was examined using TLC plate (20 x 20 cm Merck silica gel 60F254). 200 microliter of extracted aliquot was applied on the plate, parallel to the commercial lovastatin as standard (Calbiochem, 99.7% HPLC purity). The plates were made dried and dissolved in a suitable glass chamber consisting dichloromethane (Qrec) and Bendosen ethyl acetate (70:30; v/v). It was followed by observation under hand held UV (243 nm) and then exposed under iodine vapor. The retention factor (Rf) of sample and standard lovastatin was compared.

As recorded, the hybrid covered the same peak area with both authentic standards. Open ring salt of lovastatin eluted earlier than closed lactone form. A report suggested that the Rt for standard lovastatin was at 13.486 min and three different concentration of crude hybrid sample was at 13.684 min to 13.689 min reserved peak 684537.13 (mAU). The highest lovastatin production was produced which was isolated from hybrid sample extract. As reported in Table 1, the hybrid can produce a significant production of lovastatin ($p < 0.05$) up to 382.5±45.0 ug/g dry solid.

Extraction of lovastatin

The dried samples were extracted using 10 ml dichloromethane. The samples were then sonicated for5 min, followed by incubation at 30 °C with shaking speed of 200 rpm for 2 hrs and centrifuged at 3000 rpm for 8 min to separate the aliquot and biomass. 1 ml of analyte was taken out and layered with 1 % (v/v) trifluoroacetic acid for lactonization purpose. It was dried out at 80°c. Then, it was soaked in 5ml acetonitrile before been separated through 0.45 µm nylon syringe filter and then added into HPTLC [5].

Identification and characterization of hybrid from lovastatin

Infrared spectrometry

A pinch of Kbr (Potassium Bromide) pellet was oven dried and transferred into a mortar. The purified sample at about 0.1–2% was added, mixed and ground to a fine powder. The mid-range of IR spectra ranging from about 4000 to 400 cm^{-1} was recorded by a Nicolet Avatar Model FT-IR spectrophotometer [7].

Antioxidant assay

Free radical scavenging activity measurement

DPPH (2,2-diphenyl-1-picrylhydrazl) radical was determined using 1 mL of the sample with 5 mL of 0.1 mM methanol solution of DPPH followed by a vortex and incubation at 27 °C for 20 min. 0.1 mM methanol solution of DPPH alone served as the control. For negative control, 4.0 mL methanol and 1.0 mL of DPPH solution were used. The absorbance of hybrid sample was measured at 517 nm using methanol to set the blank [9].The ability of the sample to scavenge DPPH radical was calculated by the following formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{[(\text{Abs control} - \text{Abs sample}}{(\text{Abs control}) \times 100,}$$

Abs=absorbance.

Reducing power

Total reducing power was determined as described by Oyaizu et al., 1986, by adding 0.1 mL of hybrid sample solution at different concentrations with 2.5 mL of phosphate buffer (0.2 mol L^{-1}, pH 6.6) and 2.5 mL of potassium ferric amide (1%). The mixture was incubated at 50 °C for 20 min. 2.5 mL of trichloroacetic acid (TCA 10%) was added to the mixture and centrifuged at 3000 g for 10 min. The supernatant (5 mL) was mixed with 1 mL of ferric chloride (0.1%) and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates its activity.

Iron chelating activity

The iron chelating activity was measured by the decrease in absorbance at 562 nm of the iron (II)—ferrozine compel[9].. The reaction mixture contained 0.5 mL of ferric chloride (0.6 mM) and 900 µL of methanol. The mixture was shaken and left at room temperature for 10 min. To this, 0.1 mL of ferrozine (5 mM) in methanol was added, then mixed and left for 5 min to complex the residual Fe^{2+}. The control sample contained iron and ferrozine only. The absorbance of the resulting solution was measured at 562 nm. The ability of the hybrid sample to chelate ferrous ion was calculated relative to the control (consisting of iron and ferrozine only) using the formula:

Chelating effect (%)=[(Acontrol–Asample)/Acontrol]×100,A=absorbance.

Statistical Analysis

The tests hybrid samples done in sets of three to approve the test results[1]. Measurable examinations were executed with certainty cutoff points of 95% and a standard error of 0.005 utilizing the most recent SPSS 10 adaptation. The mean and SD benefits of perusing were acquired from the test duplicate. The productivity of hybrid was compared at between the monetarily accessible norm and lovastatin sample by utilizing free example 't' test and combined example 't' test and 'p' estimation of under 0.001significance was considered as measurably critical [1].

RESULTS AND DISCUSSION

Morphological and Taxonomical identification of PO3 (Hybrid)strain

The selected PO3 (hybrid) strain was characterized using standard microbiological methods such as morphological properties The organism was observed as colony morphology. The mycelium were grown on PDA plate agar medium, shown in the (Fig.1.a).white mycelium were formed and Microscopic features of hybrid mycelium via various medias (b) PDA large septate and mycelia (c) SDA medium multiple mycelia and septate (d) hyphae, branched mycelia were conjugated with clamp connection RBA medium. (Fig.1.b-d).

PRELIMINARY TEST FOR CONFIRMATION OF LOVASTATIN BY TLC

The confirmation of the lovastatin presence was carried out using thin layer chromatography, and the initial occurrence was done by comparing the Rf values of the extracts against standard lovastatin namely 0.53.

The presence of lovastatin in the extract was confirmed when the formation of dark spots on TLC plate observed under the UV light at short wave length, 243 nm where, the first spot indicates the standard form, next indicates the hybrid and the third spot indicates other parent strain. . However, the dark spots can be seen clearer under iodine vaporization (Fig.2), where the hybrid exhibits clear affinity towards the stationary phase. Commonly, qualitative screening is applied to discover a rough existence of lovastatin in fermented sample[10].This method is more suitable to disclose the polarity of compound including

lovastatin. By referring tolovastatin was a non polar compound, even though showing a slight affinity towards stationary phase.

CONFIRMATION OF LOVASTATIN BY HIGH-PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

High-performance Thin Layer Chromatography (HPTLC) is an techniques enhanced form of TLC, It is used to increase the resolution and allow to more accurate quantitative measurements.

The above figure showed the band of (right)standard lovastatin (Fig:3) and hybrid sample (left), the Rf value was calculated by TLC, when compared to standard the Rf value 0.58 was equal as hybrid sample also.The spot obtained after TLC analysis the hybrid extract was scrapped and dissolved in 1ml of ethyl acetate. Then centrifuged the mixture at 3000 rpm for 5 min, supernatant was used as High-performance Thin Layer Chromatography (HPTLC) analysis, outline showed the commercial lovastatin standard and crude hybrid sample extract was carried out by measuring the absorbance at 254nm.

HPTLC Chromatogram showed the presence of Lovastatin compound. 10mg commercial lovastatin was used as standard and 0.6µl were used as test sample. The peak was detected as blue zone by the absorbance of UV 254nm and the visible light after expose to Iodine vapours, after derivation in the chromatogram confirming the presence of Lovastatin. The peak height was given above (Fig:4.a.standard lovastatin and b.hybrid crude extracted sample).

PURIFICATION AND QUANTIFICATION OF LOVASTATIN (HPLC)

The prepared 1gm of hybrid sample extracts and a commercial lovastatin standard were quantitatively analyzed for the presence of lovastatin in the hybrid extracts. The sample wascentrifuged for 15 min at 4500rpm and filtered with filter paper and followed For HPLC, a C18 column (250 mm_4.6 mm_5 mm internal diameter) with diode array detector was used.[11]High-performance Liquid Chromatography (HPLC) analysis confirmed the presence of lovastatin in the hybrid extract.The sample was subjected into HPLC and retention time of standard lovastatin and samples were compared. The chromatogram is give below.

Quantified HPLC was carried out by using various three concentration of hybrid sample (0.5,1,1.5). The chromatogram (Fig:5.a) represents as standard lovastatin, the peak appeared 13.486. (Fig:5.b.) showed the chromatogram of hybrid crude sample on 0.5 concentration, the peak was appeared at 13.684min in (mAU) 594992.86 .then one and 1.5 concentration of hybrid sample were detected at 13.687min 610273.98(mAU) and 13.689min (Fig:5.c and d) (mAU) 684537.13. The highest lovastatin production was produced by hybrid mushroom PO3 which was isolated from hybrid mushroom sample. It can produce a significant production of lovastatin ($p < 0.05$) up to 382.5±45.0ug/g dry solid.

LOVASTATIN PRODUCTION UNDER SSF CONDITION- IN A SHAKE FLASK SYSTEM

This present experiment is believed to be the first report on the isolation, characterization and identification of a new variant of the (Hybrid mushroom) that is able to produce high amount of lovastatin in solid substrate fermentation using rice bran and brown rice as substrate. The time course profile of the lovastatin production by Hybrid mushroom in solid substrate fermentation using a combination of rice bran and brown rice (1:1 ratio) showed that the highest lovastatin production with 6.50.0±33.3 mg/g dry solid and 4.39±0.1 mg/g hybrid growth. The achievement obtained at day twelfth of fermentation day. The lovastatin and hybrid growth production decreased after achieving its maximal production. The present study showed (Fig:7.a)that solid substrate fermentation is able to produce higher yields of lovastatin and a combination of rice bran and brown rice is suitable forhybrid mushroom. The main composition of rice is starch and it is easily hydrolyzed into glucose and acetate, which is one of the compounds involved in biosynthesis of lovastatin. Furthermore, rice bran also served as good encourage medium for the mushrooms and this condition was indicated by the formation ofenhanced aerial mycelium on the substrate. It could also provide good substrate porosity for the growth of mushrooms.

Brown rice, one of anti cholesterol stars is useful in avoiding accumulation of rice bran during fermentation process. This condition has allowed an inter particle space which is very vital in macro scale mass transfer. There were many reports showed that the quantity of lovastatin production in solid substrate fermentation is significantly higher than submerged culture. compound production.

CHARACTERIZATION OF PURIFIED COMPOUND

INFRARED SPECTROSCOPY(IR)

Infrared spectroscopy is used as to identify the chemical substances or functional groups in solid, liquid or gaseous form. Here solid samples are (Fig:6.a) used for purified standard lovastatin and purified hybrid lovastatin samples. It was carried out by potassium bromide (Kbr). The mid-range of IR spectra ranging from about 4000 to 400 cm^{-1} was recorded by a Nicolet Avatar Model FT-IR spectrophotometer.

IR spectrum can be visualized in a graph of infrared light absorbance (or transmittance) on the vertical axis vs. frequency or wavelength on the horizontal axis. IR spectrum graph is represented by given below. The characteristic peaks of lovastatin (Fig:6.b)were represented at 3541.24 cm^{-1} (alcohol stretching vibration), 3023.31 cm^{-1} (olefinic C–H stretching vibration), 2335.87 cm^{-1} (methyl and methylene C–H asymmetric stretching), 1762.38 cm^{-1} (lactone and ester carbonyl stretch), 1380.09 cm^{-1} (methyl and methylene-bending vibration), 1033.88cm^{-1} (ester C–O–C symmetric bend), 832.15 cm^{-1} (trisubstituted olefinic C–H) and 750.33 cm^{-1} (meta-disturbed benzene-strong) and confirmed the presence of purified extract of Hybrid sample. Lovastatin containing the lactone ring gives characteristic peaks at 1762.38cm^{-1}

ANTIOXIDANT ASSAY

Free radical—scavenging activity measurement

The hydroxyl radical scavenging activity of the purified extracts of hybrid was dose dependent. At a concentration of 199.2 µg mL^{-1} (200 µL), the scavenging activity of purified lovastatin was optimum and it is almost nearly equal to that of the standard lovastatin as shown in the (Fig. 8 a), The IC_{50} of the purified lovastatin was at 148.2 µg mL^{-1} (140 µL) demonstrating the inhibitory activity and it was not higher than the standard.

Reducing power

Reducing power reflects the antioxidant activity significantly. The reducing capacity of the purified hybrid extract was observed maximum at 120.3 µg mL^{-1} (120 µL) and also optimum at 152.01 µg mL^{-1} (140 µL) and 170.05 µg mL^{-1} (160 µL). The reducing power of the purified extracts is also represented in (Fig. 8.b)

Iron chelating activity

The iron chelating assay was used to evaluate the ability of purified lovastatin to disrupt the complex formation. In experiment, it was observed that the percentage of inhibition is maximum at 180.1 µg mL^{-1} (180 µL) and 199.5 µg mL^{-1} (200 µL) which is statistically significant ($p < 0.0001$). At other doses also, it is statistically significant ($p < 0.05$) in case of both test sample (hybrid extract) and standard (Fig.8.c).

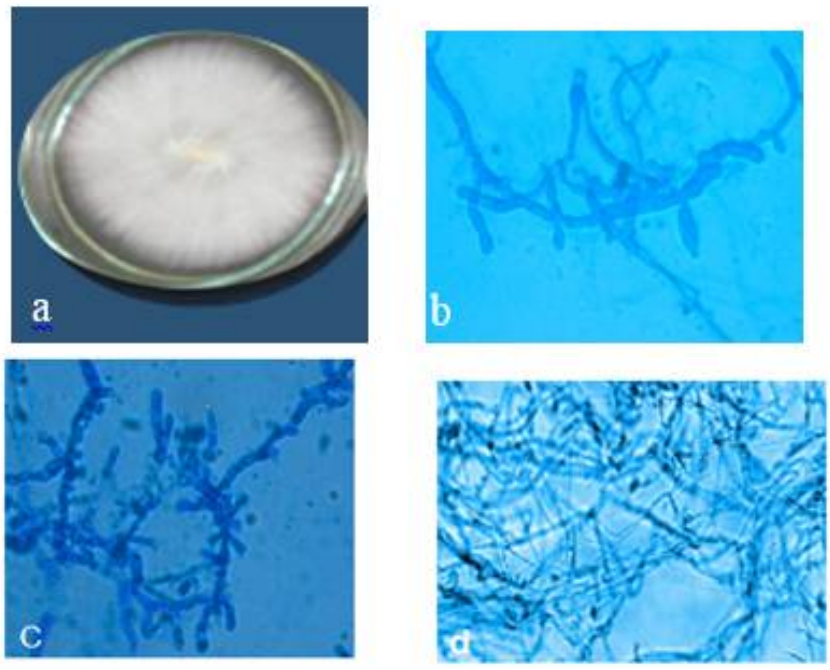

Figure 1:Macroscopic & Microscopic features of hybrid mycelium via various medias (a) PDA Plate (b) PDA large septate and mycelia (c) SDA medium multiple mycelia and septate (d) hyphae, branched mycelia were conjugated with clamp connection RBA medium.

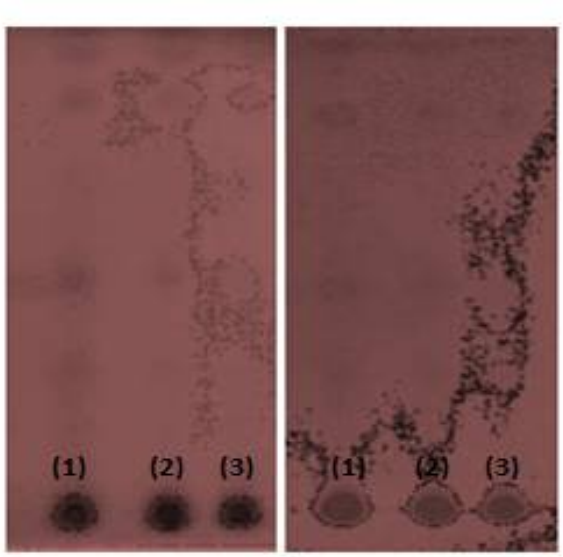

Figure 2: The confirmation of the lovastatin presence was carried out using thin layer chromatography. (a) Dark spots on TLC in fermented sample under uv at 254nm (b) Apperance after vaporized with Iodine

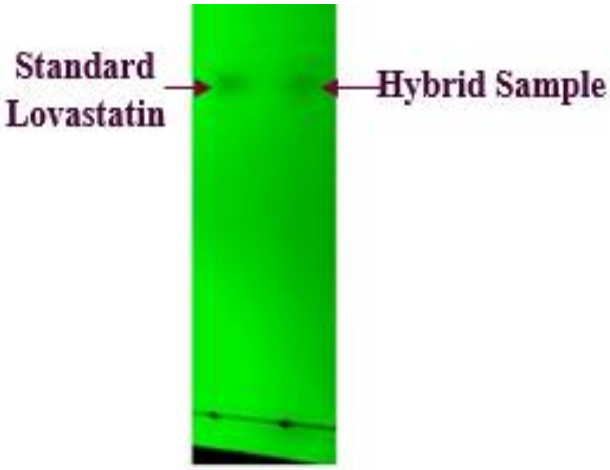

Figure 3 : Determination of Lovastatin from hybrid sample by HPTLC

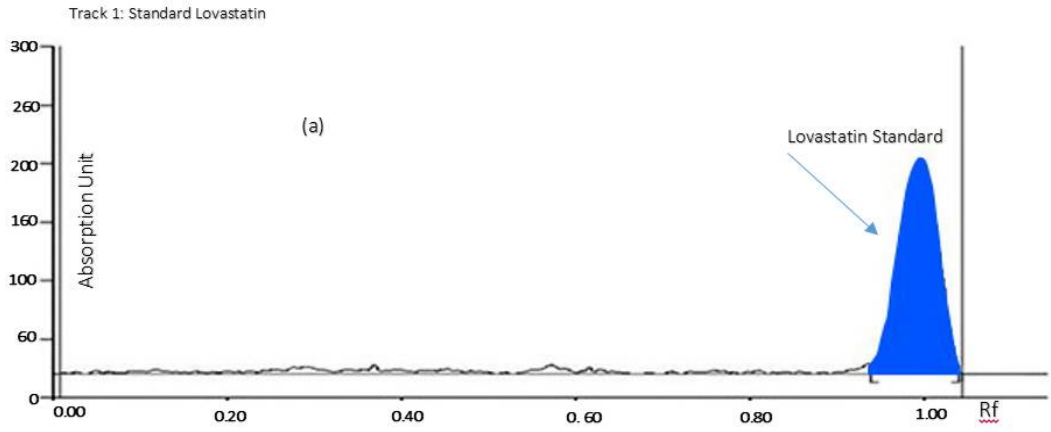

Figure 4.a: Standard Lovastatin were measured at 254 nm absorbance

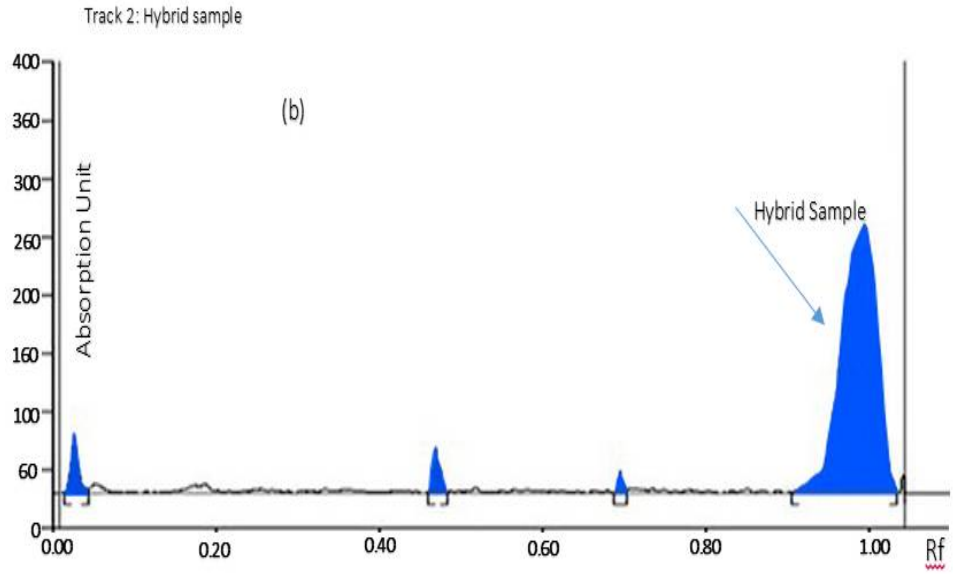

Figure4.b: Hybrid crude extracted lovastatin was carried out by measuring the absorbance at 254 nm

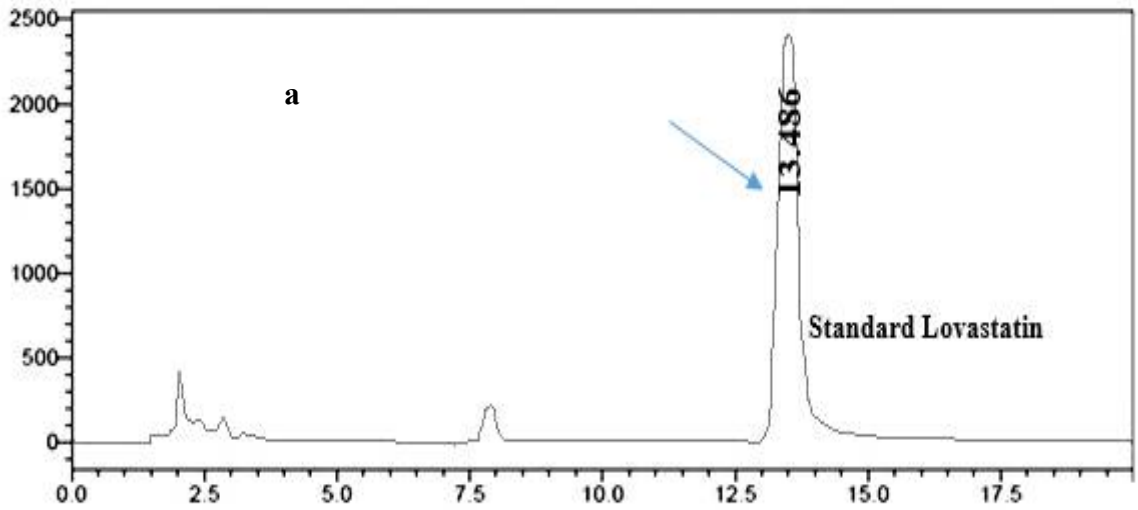

Figure 5.a: HPLC chromatogram of standard Lovastatin at 13.486min

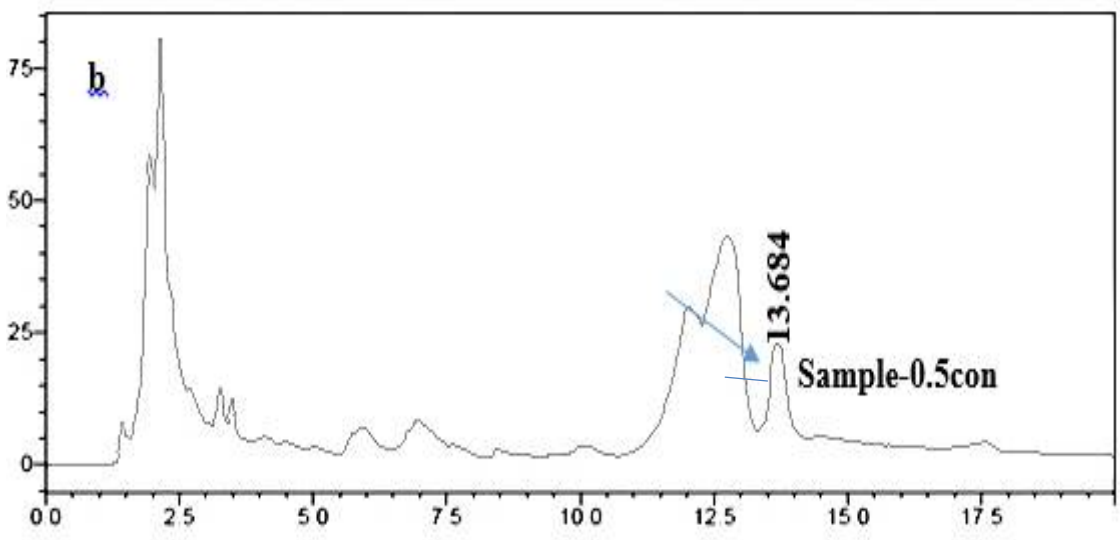

Figure 5.b: HPLC chromatogram of Hybrid sample 0.5 concentration at 13.684min

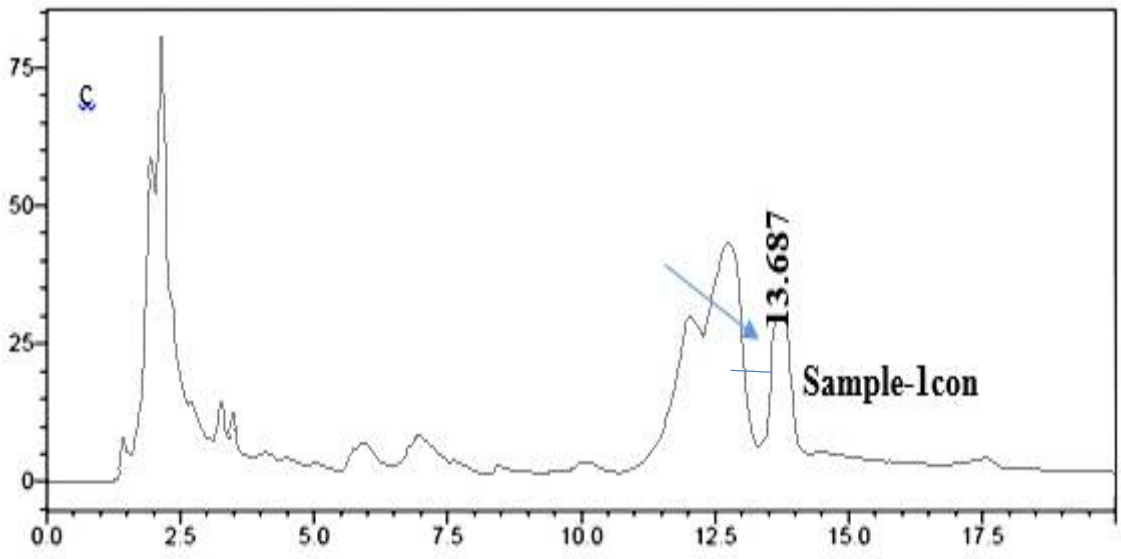

Figure 5c: HPLC chromatogram of Hybrid sample 1 concentration at 13.687 min

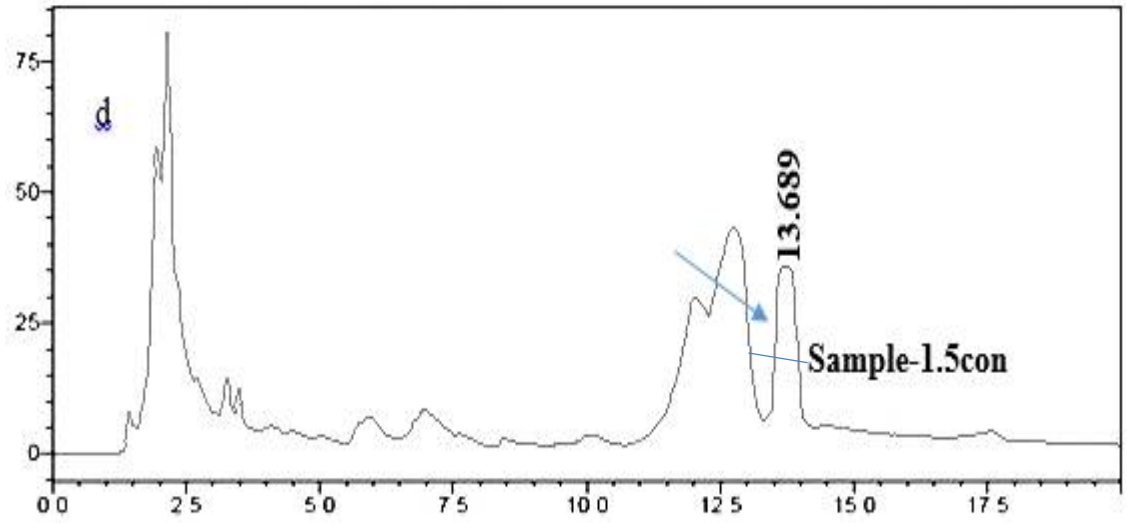

Figure 5.d: HPLC chromatogram of Hybrid sample 1.5 concentration at 13.689 min

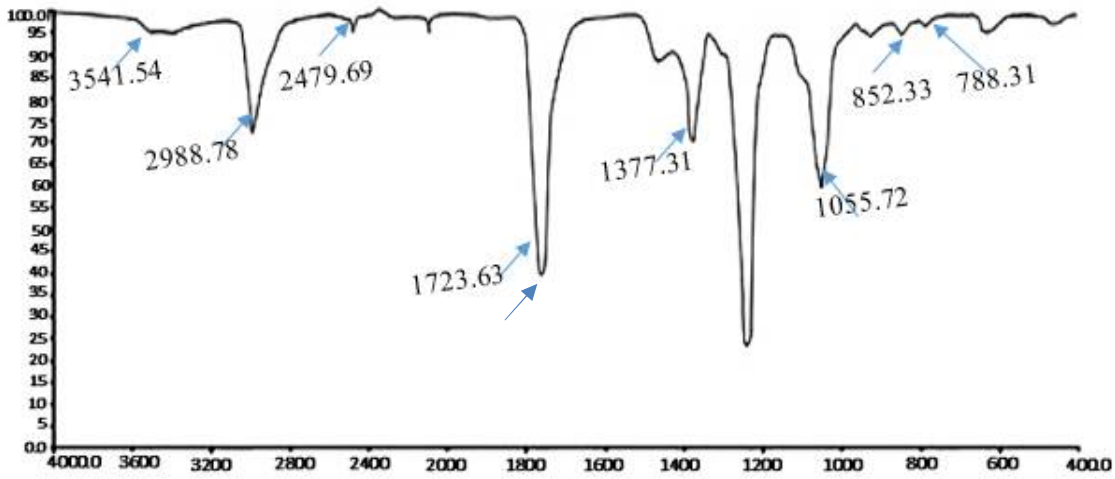

Figure 6.a: Purification of standard lovastatin compound by using IR spectrum

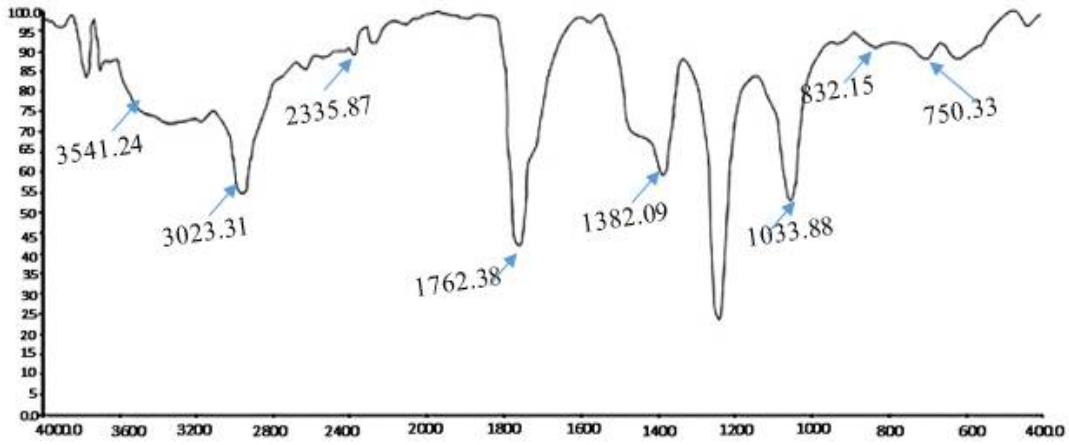

Figure 6.b: Characterization of purified lovastatin compound from hybrid by using IR spectrum

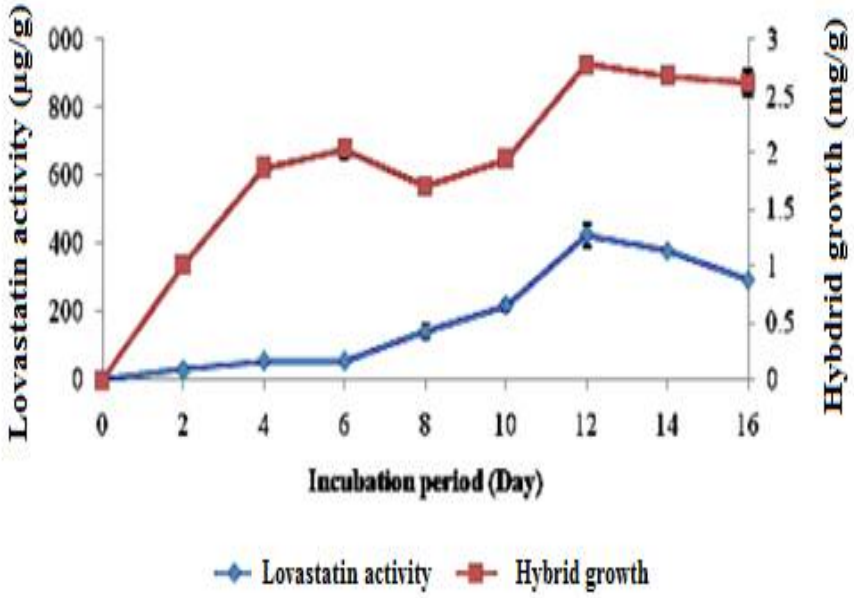

Figure 7.a: Production of Lovastatin by Hybrid growth extracts

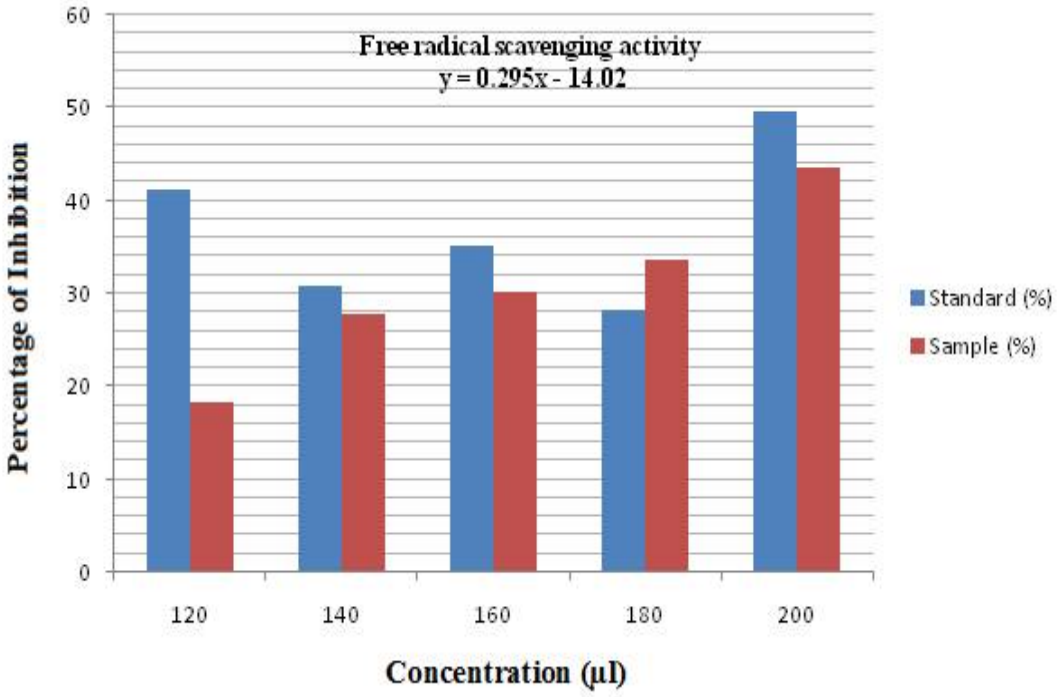

Figure 8.a: Assessment of Free radical—scavenging activity using DPPH

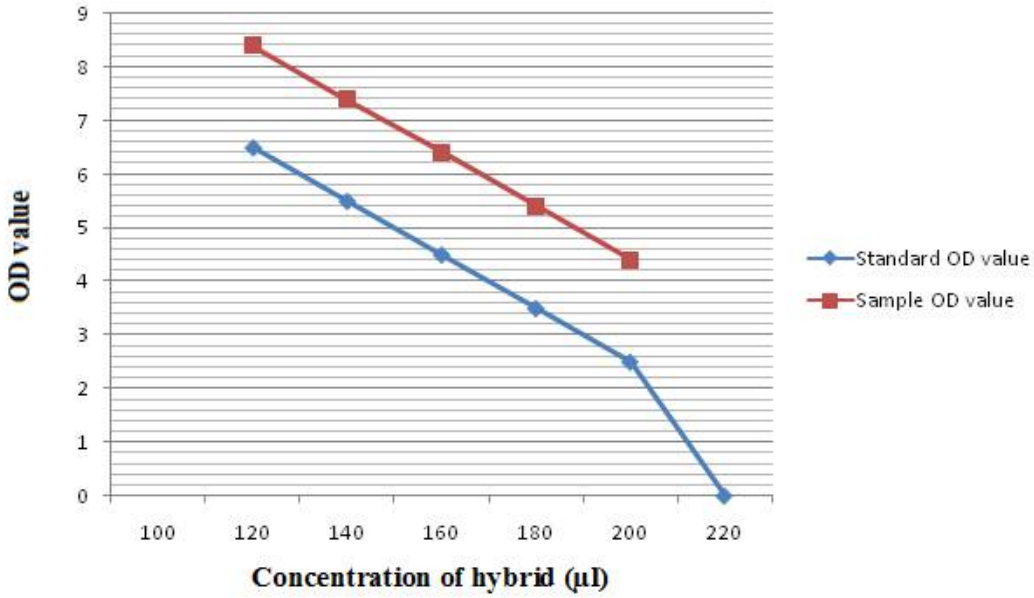

Figure 8.b: Reducing power evaluation of lovastatin frompurified extract

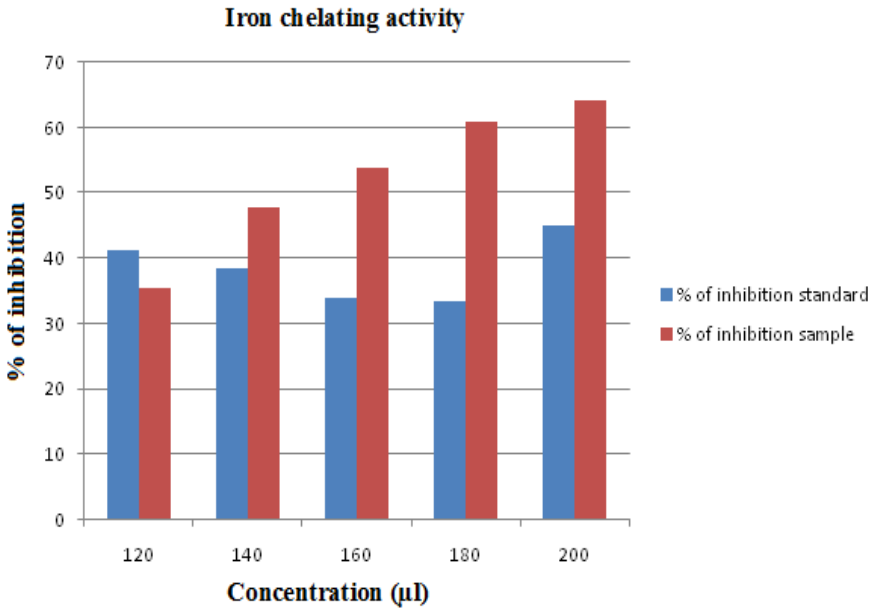

Figure 8.c : Assessment of Iron chelating ability of standard lovastatin and purified sample

CONCLUSION

The confirmation of the lovastatin presence was carried out using TLC and HPTLC methods, Presence of lovastatin in the extract was confirmed when the formation of dark spots and observed under the UV light at short wavelength, 243 nm. However, dark spots can be seen clearer under iodine vaporization. After extraction of lovastatin from hybrid, they can be even characterized for identification of pure bioactive compounds, which can be done by High Performance Liquid chromatography and IR spectrometry. analysis can be done to identify the growth of hybrid on solid media with mineral variations can be marked for standards of soluble lovastatin analysis. Antioxidant and reducing power evaluation of purified lovastatin, assessment of free radical scavenging activity using DPPH were obtained from hybrid. Commonly, qualitative screening is applied to discover a rough existence of lovastatin in fermented sample. This method is more suitable to disclose the polarity of compound including lovastatin. Thus, the isolation, screening and analysis of lovastatin level were obtained in hybrid.

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