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Endophytic Fungi: A Poor Candidate for the Production of Lovastatin

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Authors' contributions

This work was carried out in collaboration between all authors. Author JS designed the study and composed the protocol. Authors VKP and SDB managed the literature searches and analysis aspect. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: The aim of the present study was to screen soil and endophytic fungi for production of lovastatin.
Methodology: Soil fungi were isolated by dilution plating technique and endophytic fungi from selected medicinal plants by using standard procedures. All isolates were tested for lovastatin production by Solid State Fermentation (SSF) using wheat bran as substrate.
Results: The soil isolate, *Aspergillus terreus* NCBI (KM017963) showed positive for lovastatin (1.0 mg/G DWS) whereas none of the endophytic fungi tested showed positivefor lovastatin production.

Keywords: Aspergillus terreus; endophytic fungi; Lovastatin; soil fungi.

1. INTRODUCTION

Lovastatin, a fungal secondary metabolite, acts as one of the competitive inhibitors of the enzyme hydroxyl methyl glutaryl coenzyme A (HMG-CoA) reductase, which catalyses the conversion of HMG CoA to mevalonate during cholesterol biosynthesis. Because of this nature, lovastatin finds its role in biomedical applications such as, in treatment of coronary heart diseases, renal diseases, Alzheimer's disease, bone fractures etc. Lovastatin has also been used as a potential therapeutic agent for the treatment of various types of tumors

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because of its ability to suppress tumor growth *in-vivo* [1]. The antifungal activity of various statins against yeasts are also reported for *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*, *Cryptococcus neoformans*and *Saccharomyces cerevisiae* [2].

Several fungal genera including *Aspergillus, Penicillium, Monascus, Paecilomyces, Trichoderma, Scopulariopsis, Doratomyces, Phoma, Pythium, Gymnoascus* and *Hypomyces* are reported as lovastatin producers. However, only *Aspergillus terreus* has thus far been used in commercial production [3,4]. A marine Actinomycete has also been reported to produce lovastatin [5]. It is also naturally produced by certain higher fungi such as, *Pleurotus ostreatus* (oyster mushroom) and closely related *Pleurotus* species. Although, the potential of different classes of fungi to produce lovastatin is well documented, none of them have been utilized in commercial scale production. Commercial lovastatin production was carried out by Submerged Fermentation (SmF) and of-late its production using Solid State Fermentation (SSF) is being investigated due to several advantages [6,7,8].

Endophytic fungi, the organisms that inhabit healthy living tissue of plant without causing any harm, have been widely studied for their ability to produce an array of secondary metabolites which are of commercial importance. Although it is well documented that secondary metabolites produced by endophtyes possess anticancer (Paclitaxel), antibiotic (Cryptocandin), anti-oxidant (Pestacin), immunosuppressive (Subglutinol A and B) and antidiabetic (2,6-di-tert-butyl-p-cresol) properties[9] , there is no report available till date on the production of anti-cholesterol drug, lovastatin. Therefore, an attempt was made in the present study to evaluate the ability of endophytic fungi to produce lovastatin and, if produced, their suitability for solid state fermentation.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of Soil Fungi

Soil samples were collected in sterile containers from various regions of Bangalore, Karnataka, India and serially diluted up to $10^{-6.}$ The serially diluted soil samples were plated onto Potato Dextrose Agar (PDA) supplemented with 50mg/L tetracycline to suppress the bacterial growth and incubated at 37ºC for 3-4 days until fungal growth was noted. Each isolate was sub-cultured and maintained on PDA slants [10]. Fungal identification was done as described by Subramanyam, 1982 [11].

2.2 Isolation and Identification of Endophytic Fungi

The following medicinal plants *Piper nigrum, Garcinia cambogia, Commiphora wightii, Coleus aromaticus,Vetiveria zizanoides, Curcuma amada, Patchouli pogestemon and Terminalia arjuna* were collected from University of Agricultural Sciences (UAS) GKVK, Bangalore and Irinjalakida town, Thrissur Dist, Kerala, India. Various parts of fresh plants (stem, leaf, roots) were cut into small pieces using sterile blade and washed with sterile distilled water. The samples were then surface sterilized by dipping into 4% sodium hypochlorite for 60 seconds and 70% ethanol for 5 seconds, rinsed with sterile water and allowed to dry under sterile conditions. The samples were placed on Potato Dextrose Agar (PDA) plates amended with 50mg/L tetracycline to suppress the bacterial growth and incubated at 28ºC - 30ºC for 2 to 3 days. The hyphal tip of endophytic fungi growing out from

the plant tissue was transferred to fresh PDA plates amended with 50mg/L tetracycline [12]. Endophytes were identified as described by Barnett and Hunter., 1982 [13].

2.3 Solid State Fermentation

Wheat bran was used as a sole source of substrate for lovastatin production. Two grams of substrate was weighed and relative humidity was maintained at 70%. One milliliter of spore suspension (10^6 spores) was added to the sterilized substrate and incubated at 28ºC [14].

2.4 Extraction

After ten days of incubation, the solid substrate was dried at 40ºC for 24 hrs, crushed and extracted with 10ml ethyl acetate by shaking at 180 rpm for 2 hrs followed by filtration through Whatman No. 1 paper. To 1ml of extract, 1ml of 1% trifluroacetic acid was added and incubated for 10 minutes (lactonization of hydroxyl acid form of lovastatin). The filtrate was then spotted onto Thin Layer Chromatography (TLC) for detecting the presence of lovastatin in crude extract [15].

2.5 Thin Layer Chromatography

The extracted organic phase was concentrated to about 50µl using a block heater adjusted to 45ºC, applied to a heat activated 20 × 20cm silica gel TLC plates. Dichloromethane and ethyl acetate (70:30, v/v) were used as mobile phase. All the plates were observed under a hand-held UV lamp (254nm) after developing three times in the same mobile phase and stained afterward with iodine vapour. For each TLC run, lovastatin authentic standard (Merck) was also included for Rf value comparison and confirmation [16].

2.6 High Performance Liquid Chromatography (HPLC)

For HPLC, a ODS (250mm x 4.6mm I.D micro metre) column with diode array detector was used. Acetonitrile and phosphoric acid (60:40 v/v) were used as mobile phase. The eluent flow rate was maintained at 1.5ml per minute and detection carried out at 238nm with injection volume of 20 µl[14]. The production of lovastatin is expressed in µg/G dry weight substrate (DWS). The yield of lovastatin was calculated according to the published method [17]. Mevinolin (M2147) (Sigma-Aldrich, Germany) was used as standard.

2.7 Molecular Identification

Fungi were grown in 50 ml Potato Dextrose Broth for 5-6 days at 28ºC. The mycelia was harvested and washed with distilled water and ground with liquid nitrogen. The nucleic acid was extracted using Cetyltrimethyl ammonium bromide (cTAB) method. Polymerase chain reaction was done using universal primers ITS1 and ITS4. BLAST analysis was done with available NCBI database and the sequences submitted to NCBI [18].

3. RESULTS AND DISCUSSION

Lovastatin, a pharmaceutically important chemical compound has long been used for the treatment of hypercholesterolemia. It is a secondary metabolite produced during the secondary phase of fungal growth. The soil fungus *Aspergillus terreus* which was originally discovered as lovastatin producer in 1979 has been used in the commercial production of

lovastatin after obtaining clearance from Food and Drug Administration (FDA) in 1987 [19]. This is the only fungal isolate that has been utilized in the commercial level production of lovastatin.

Continued search for microorganisms may offer potential candidates that could produce still higher levels of lovastatin. Therefore, there is a need to explore other microbial habitats such as, those living internal tissues of plants for novel and potent microorganisms. Fungal endophytes (i.e) the fungi growing within plant tissues without causing any signs of harmful symptoms or disease, are known to produce diverse bioactive secondary metabolites. These metabolites exert bioactivity against cancer cell lines, pathogenic bacteria, fungi and also against eukaryotic parasites such as Malaria, Leismaniasis and Chagas disease [20]. It is well documented that the metabolites produced by endophytes generally exhibit diverse range of structures including alkaloids, terpenoids, quinones, xanthones and phenols [21]. Therefore, the present study was undertaken to screen available endophytic fungi from medicinal and soil fungi for their ability to produce lovastatin.

The procedures used for the isolation of fungi from plants and soil yielded fungal colonies that were purified to obtain pure cultures. Of the fifty four endophytic fungi isolated from eight different medicinal plants (Table 1), 41% belonged to the genus *Aspergillus,* 12.5% to *Penicillium*, 17.8% to *Fusarium*, 8.92% to *Cladosporium, 3.5% to Phomopsis, 3.5 % to Pestalotiopsis, 3.5% to Collectotrichum* and 8.9% to the group mycelia sterilia.

Among the 360 fungi isolated from soil, *Aspergillus* spp were dominant (50%) followed by *Penicillium* spp. (25%). The remainder of the proportion belonged to the genus *Fusarium, Trichoderma*, *Rhizopus*, *Mucor, Cunninghamella*with a few sterile mycelia (Table 2).All fungal isolates were screened for lovastatin production with wheat bran as solid substrate under the conditions described in the methods section. At the end of the incubation period, the cultures were processed for lovastatin extraction and HPLC analysis.

Few species of *Aspergillus* (180 isolates) produced varying levels of lovastatin as indicated in Table 2. Of the fifteen *A terreus* strains isolated, three strains of *Aspergillus terreus* i.eL1, L2 and L3 produced 1.0mg/G DWS (Dry Weight Substrate), 0.680 mg/G DWS and 0.330 mg/G DWS of lovastatin, respectively and *A terreus* L 1 which showed better yield (Fig. 2b) was taken for further study.

Aspergillus terreus L 1 was identified by molecular methods. The sequence was submitted to NCBI (accession number KM017963). Thin Layer Chromatography analysis revealed the presence of UV fluorescent spot at an Rf value of 0.67 which coincided with the authentic lovastatin indicating the presence of lovastatin in *A. terreus* L 1 (NCBI-KM017963) extracts (Fig. 1).

Our results are in agreement with published reports that *Aspergillus terreus* is the best producer of lovastatin [3,19]. According to Jaivel and Marimuthu, and Raghunath et al., the yield of Lovastatin in a strain of *A terreus* JPM3 and *A niger* similar such yield i.e 0.980mg/G DWS [14,22]. Although the substrates in which they were grown were different.

Table 1. List of endophytic fungi isolated from medicinal plants

No.	Plant source	Part of plant	Organism
1	*Piper nigrum*	stem	*Aspergillus* sp
			Fusarium sp
			Aspergillus sp
			Penicillium sp
			Phomopsis sp
		leaf	*Aspergillus* sp
			Penicillium sp
		root	*Aspergillus* sp
			Penicillium sp
			Fusarium sp
			Aspergillus sp
2	*Garcinia cambogia*	stem	*Cladosporium* sp
			Aspergillus sp
		root	*Fusarium* sp
			Aspergillus sp
			Pestalotiopsis sp
3	*Commiphora wightii*	stem	*Fusarium* sp
			Aspergillus sp
		root	*Fusarium* sp
			Fusarium sp
			Aspergillus sp
			Phomopsis sp.
4	*Coleus aromaticus*	stem	*Aspergillus* sp
		leaf	*Aspergillus* sp
		root	*Penicillium* sp
			Aspergillus sp
5	*Vettiveria zizanoides*	leaf	*Aspergillus* sp
			Cladosporium sp
			Sterile mycelium
			Fusarium sp
			Pestalotiopsis sp
6	*Curcuma amada*	stem	*Sterile mycelium*
			*Cladosporium*sp
			Aspergillus sp
		leaf	*Cladosporium* sp
			Sterile mycelium
			Fusarium sp
			Aspergillus sp
7	*Patchouli pogestomon*	stem	*Penicillium* sp
			Penicillium sp
			Aspergillus sp
		leaf	*Penicillium* sp
			Aspergillus sp
			Aspergillus sp
			Aspergillus sp
			Colletotrichum sp
8	*Terminalia arjuna*	stem	*Aspergillus* sp
			Aspergillus sp
			Fusarium sp
			Colletotricum sp
		leaf	*Fusarium* sp
			Cladosporium sp
			Aspergillus sp
			Aspergillus sp

Table 2. Production of lovastatin by soil fungi

No.	Genus	Number of isolates	Isolate	Yield mg/G DWS
1	*Aspergillus* sp	180	*Aspergillus terreus*(L1)	1.0
			Aspergillus terreus(L2)	0.680
			Aspergillus terreus (L3)	0.330
			Aspergillus sp (L4)	0.220
			Aspergillus sp(L5)	0.180
			Aspergillus sp(L6)	0.150
2	*Penicillium sp*	90	---	---
3	*Fusarium sp*	18	---	---
4	*Trichoderma sp*	25	---	---
5	*Cunninghamella sp*	4	---	---
6	*Rhizopus sp*	18	---	---
7	*Mucor sp*	18	---	---
8	Sterile mycelia	7	---	---

Fig. 1. TLC of extracts: Lane 1: *Aspergillus terreus*; soil isolate (NCBI KM017963); Lane 2: Lovastatin standard: Lane 3: Endophtyic fungi extract

HPLC analysis of the extracted lovastatin in comparison with the authentic lovastatin (Fig. 2a) showed the presence of prominent lovastatin peak in the HPLC profile at 11.4 min (retention time) in addition to other metabolites that was present in the crude extract.

It is of interest that none of the 54 endophytic fungi isolated from medicinal plants and screened showed detectable levels of lovastatin production even after 10 days of incubation (Fig. 1 and Fig. 2c). Whether or not the lack of lovastatin production by all endophytic fungi screened in this study is a general feature of those isolated from medicinal plants remains to be seen. While these results enable us to conclude most, if not all, endophytic fungi may not be the potential candidates for lovastatin production, the results are of considerable importance in view of their endophytic (symbiotic) relationship with their host plants.

Fig. 2a

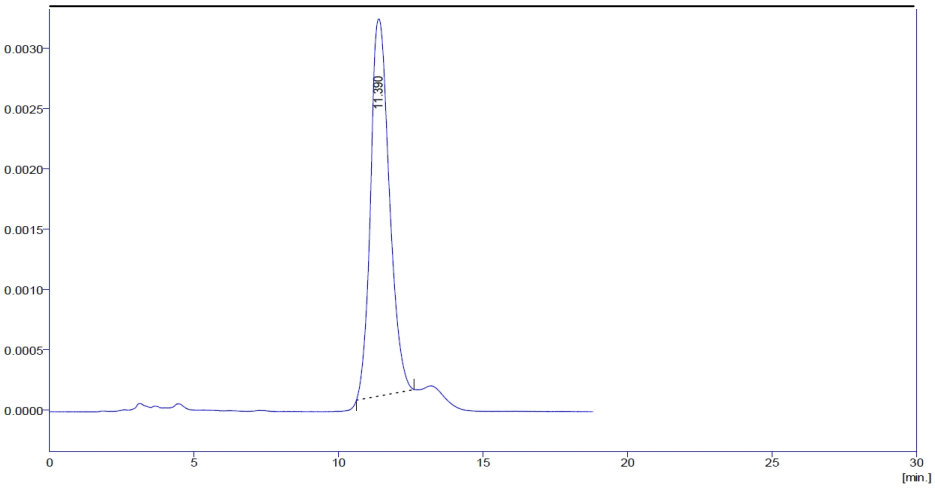

Fig. 2a. Lovastatin standard

Fig. 2b

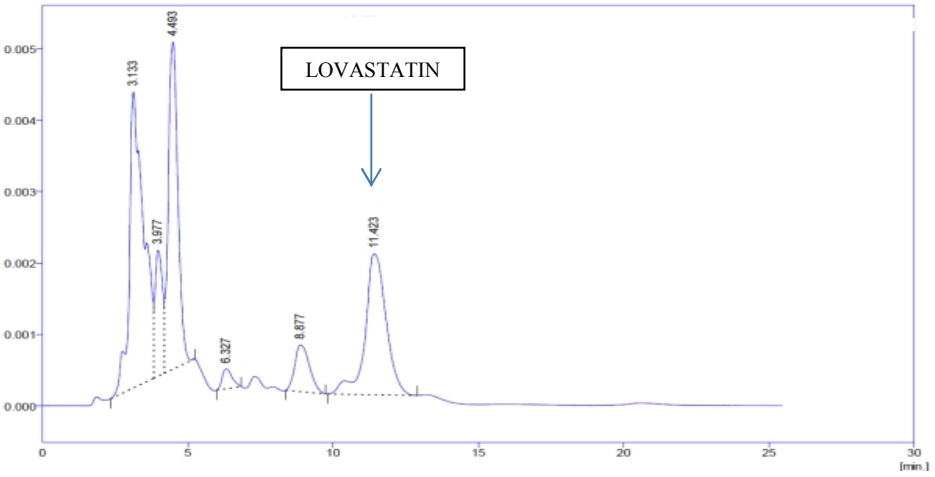

Fig. 2b. *Aspergillus terreus* L1 (NCBI-KM017963): A soil isolate

Fig. 2c

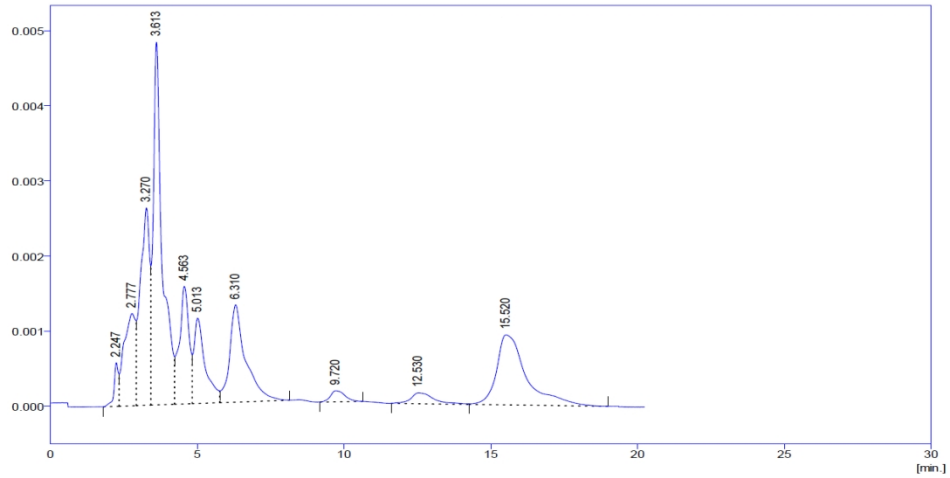

Fig. 2c. *Aspergillus* sp. isolated from *Piper nigrum* (an endophyte)
Fig. 2. HPLC analysis of lovastatin

Lovastatin is a known inhibitor of the enzyme, HMG CoA reductase, an enzyme involved in cholesterol biosynthesis and thus elicited interest in biomedical applications. In plant systems on the other hand, HMG CoA reductase inhibits the synthesis of plant growth promoting substances such as ABA, gibberellins, ubiquinone, carotenoids and isopyrenoids [23,24] by the inhibition of mevalonate production. Indeed, the inhibition of HMG CoA reductase and growth of Tobacco cells was observed by low concentrations of lovastatin and reversal of such inhibition by Cytokinin has also been reported [25].

Endophytes are microorganisms that are endo-symbionts residing in plants at least during part of their life cycle without any harm [26]. In view of the fact that none of the fifty four endophytic fungi isolated from medicinal plants and screened in this study produced detectable levels of lovastatin combined with the fact that the inhibition of plant growth by lovastatin [25], the lack of lovastatin production by endophytic fungi appears to solely not to harm the plant host system they are residing in.

4. CONCLUSION

Terrestrial species of *Aspergillus, Penicillium*, *Monascus*, *Paecilomyces, Trichoderma, Scopulariopsis, Doratomyces, Phoma,* produce lovastatin while endophytic species of the same genera do not, indicating that these endophytic fungi may not have necessary genetic machinery to produce lovastatin or regulated differently. While the endophyte/plant relationships are not clearly understood as compared to typical symbionts, the present study has thrown some light towards the understanding of endophytic fungi and medicinal plant relationship.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Sreenivasan A, Shubahgar S, Arvindan R, Viruthagiri T. Microbial production and biomedical applications of Lovastatin. Indian J Pharma Sci. 2008;70:701-9.
2. Laszlo GI, Nyilas, Papp T, Vagvoljy C. Statins as antifungal agents. World J Clinical Infectious Dis. 2011;30:(1):4-10.
3. Marcin B, Stanislaw L. Physiological, morphological and kinetic aspects of lovastatin biosynthesis by Aspergillus terreus. Biotech J. 2009;4:1-61.
4. Osman ME, Khattab OH, Zaghlo GM, Rehab MAE. Screening for the Production of Cholesterol Lowering Drugs (*Lovastatin*) by Some Fungi. Aus J Basic Appl Sci. 2011;5:698-703.
5. Srinu M, Bhushan GVP, Moges F, Srilakshmi J, Sankar G, Prabhakar T, Lakshminarayana K. Screening of HMG CoA reductase inhibitor producing marine actinomycetes. J Pharm. Sci. Health. 2010;2:66-74.
6. Panda BP, Javed S, Ali M. Engineering Rice Based Medium for Production of Lovastatin with *Monascus species*. Czech J. Food Sci. 2009;27:52–360.
7. Latha PM, Chanakya P, Srikanth M. Lovastatin by Aspergillus fischeri under solid state fermentation from coconut oil cake. Nepal J Biotec. 2012;2:26-36.
8. Praveen VK, Savitha, J. Solid State Fermentation: An Effective Method for Lovastatin Production by Fungi – A Mini Review. The Open Tropical Medicine J. 2012;5:1-5.
9. Padhi L, Yugal, Mohanta YK, Panda SK. Endophytic fungi with great promises: A Review. J Adv. Pharm. Edu. & Res. 2013;3:52-170.
10. Aneja K R, Experiments in Microbiology, Pant pathology and Biotechnology, Newage International Publishers. 2001;4:157-162.
11. Subramanian CV. Hyphomycetes: Taxonomy and Biology. Academic Press Inc; 1982.
12. Nithya K, Muthumary J. Secondary Metabolite from *Phomopsis Sp*. isolated from Plumeriaacutifolia Poiret. Recent Res Sci Tech. 2010;2:99-103.
13. Barnett, HC, Hunter, BB. Illustrated genera of imperfect fungi. 1982. 3. ed. Mineapolis.
14. Jaivel N, Marimuthu P. Optimization of lovastatin production in solid state fermentation by Aspergillus terreus. Intl J Engg. Sci. Technol. 2010;2:2730-2733.
15. Reddy SR, Latha DP, latha KPJ. Production of Lovastatin by Solid State Fermentation by Penicilliumfuniculosum NCIM 1174.Drug Invention Today. 2011;3:75- 77.
16. Samiee SM, Moazami N, Haghighi S, Mohseni FA, Mirdamadi S, Bakhtiari MR. Screening of lovastatin production by filamentous fungi. Iran Biomed Journal. 2003;7:29-33.
17. Muthumary J, Sashirekha S. Detection of taxol, an anticancer drug, from selected coelomycetous fungi. Indian J SciTechn. 2007;1:1-10.
18. Upendra RS, PratimaKhandelwal, Z R Amiri, Shwetha L and Ausim M. Screening and Molecular Characterization of natural fungal isolates producing Lovastatin. Microbial & Biochemical Technology. 2013;5(2):025-030.

19. Barrios GJ, Miranda RU. Biotechnological production and applications of statins. Appl. Microbiol. Biotech. 2010;85:869-883.
20. Rosa LH, et al, Leishmanicidal, trypanocidal, and cytotoxic activities of endophytic fungi associated with bioactive plants. Brazil J Microbio. 2010;41:420-430.
21. Zhao JH, Zhang YL, Wang JY, Wang CL, Zhang. Bioactive secondary metabolites from *Nigrospora sp.* LLGLM003, an endophytic fungus of the medicinal plant Moringaoleifera Lam. World J Microbiol Biotechnol. 2012;28:2107.
22. Raghunath R, Radhakrishna A, Manikandan N, Nathiya K, Palaniswamy M. Optimised production of lovastatin through solid state fermentation by endophytic fungi. Int'l J Pharma Bio Sci. 2012;3(3):(B)562-570.
23. Kakimoto T. Biosynthesis of cytokinins. J Plant Res.1984;116:233–239
24. Goldstein, Brown. Regulation of the mevalonate pathway. Nature. 1990;1;343(6257):425-30.
25. Crowell DN, Salaz MS. Inhibition of growth of cultured Tobacco cells at low concentrations of Lovastatin is reversed by Cytokinin. Plant Physiol. 1992;100:2090-2095.
26. Strobel Daisy. Bio prospecting of microbial endophytes and their natural products. Microbio. Mol. Boil. Rev. 2003;67:491-502.

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