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Bioprospecting medicinal plants for the isolation and screening of lovastatin producing endophytic fungi

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Endophytic fungi reside within the plant tissues asymptomatically and produce various secondary metabolites of pharmaceutical interest. The current study aims to bio-prospect medicinal plants for the isolation and screening of lovastatin producing endophytic fungi. Endophytic population in the leaf, stem, root and flower (as applicable) of ten medicinal plants has been studied and their potential to produce the anti-hypercholesterolemia drug, lovastatin has been evaluated. A total of 98 fungal isolates were obtained from the plant tissues and lovastatin yield from them was quantified to be within the range of 5 mg/L to 71.5 mg/L in the first round of submerged fermentation. The subsequent levels of screening witnessed a great change in the yield which could be attributed to gene attenuation, a usual phenomenon in endophytes. A novel lovastatin producer, designated as HL1, belonging to *candida sp.* residing within the leaves of *Hibiscus rosa-sinensis* was found to consistently yield higher amounts of lovastatin i.e., 40 mg/L through all rounds of screening, alongside two strains of *Aspergillus sp.*, designated as HL4 and HL5, from the same tissue with a yield of 21.5 and 18 mg/L respectively. Preliminary confirmation of lovastatin presence in the fungal extract was done by Thin Layer Chromatography (TLC) and yeast growth inhibition bioassay.

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Introduction

The use of natural products in medicine dates back to ancient times. Natural products are secondary metabolites produced by various organisms as a defence mechanism in response to stress[1]. The natural products themselves or their principal molecules are potential candidates for drug development[2]. Globally, about 50% of the novel drug molecules approved for marketing between 1981 and 2002 were of natural origin[3]. However, exploitation of the natural products for the drug discovery has declined over the last few decades owing to the advent of combinatorial chemistry, metagenomics and high-throughput screening. Plants apart, microorganisms are an excellent source of metabolites with bioactive properties. Among the microorganisms, fungi, the most important group of eukaryotes are very well known for their ability to produce metabolites of clinical significance[4]. In the 1990s, around 1500 fungal metabolites have been reported to possess anticancer and antibiotic properties[5].

Various soil fungi have been shown to produce lovastatin, the first statin approved by the FDA as an anti-hypercholesterolemia drug which eventually found its application in the treatment and management of various other chronic diseases including one of the global health challenges, cancer[6]. Other ecological habitats such as plant tissues have been less exploited for the isolation of potent lovastatin producers[7, 8].

This study aims to bio-prospect medicinal plants for the isolation and screening of lovastatin producing endophytic fungi. The mysterious existence of endophytic fungi for varying periods in the plant tissues and the consequent production of secondary metabolites by them in response to the stress induced by the host are of immense interest to the bio-prospectors. They seem to be metabolically aggressive and versatile as compared to their counterparts in other niches since they ought to maintain a constant interaction with their host encountering the host defences and withstand the selection process through novel metabolic pathways[9]. The present study aims to exploit this potential of endophytic fungi by isolating them from the

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medicinal plants and screening for the production of lovastatin, one drug with many applications.

Endophytic colonization is believed to benefit their host plant in three ways – enhancing the growth of host plant, conferring the plant with resistance to biotic and abiotic stresses and producing bioactive compounds that can be used as drugs[10]. The secondary metabolites produced by the endophytic fungi could be originally from plants as in the case of paclitaxel[11], camptothecin, and its structural analogs[12-14] or those that were only produced by the host plants such as Artemisinin[15]. The ability of the endophytes to synthesize the same natural products as the host plant could be apparently due to horizontal gene transfer or genetic recombination between the host and the endophytes it harbors[13]. Nevertheless, the exact nature of the interaction between the endophytic microbe and the plant, plant products and processes involved are yet to be elucidated[10].

The rationale behind selecting medicinal plants as the source for bio-prospecting is that endophytic fungi associated with the medicinal plants were found to be promising sources of metabolites[16-20]. Besides, plants growing in unique environments and those with exceptional biology are also worth exploiting[21]. Different tissues of the selected host plants have been screened for endophytes rather than similar tissue from different plants since the endophytes were found to be tissue-specific rather than taxon-specific[22,23].

To the best of our knowledge, for the first time, novel lovastatin producing yeast belonging to *Candida* sp. residing within the leaves of *Hibiscus rosa-sinensis* was identified in this study. It is noteworthy that this strain exhibited consistency in its lovastatin production across two levels of screening with a yield of 40 mg/L under submerged conditions despite gene attenuation that occurs in endophytes as a result of continuous sub-culturing.

Materials and Methods

Sample collection

Based on their therapeutic properties, ten medicinal plants namely *Ocimum tenuiflorum*, commonly known as holy basil (Family: Lamiaceae), *Emblica officinalis*, commonly known as gooseberry (Family: Phyllanthaceae), *Moringa oleifera*, commonly known as drumstick (Family: Moringaceae), *Bacopa monnieri*, commonly known as water hyssop (Family: Plantaginaceae), *Murraya koenigii* commonly known as curry leaf (Family: Rutaceae), *Azadirachta indica*, commonly known as neem (Family: Meliaceae), *Hibiscus rosa-sinensis*, commonly known as China rosa (Family: Malvaceae), *Catharanthus roseus*, commonly known as periwinkle (Family: Apocynaceae), *Withania somnifera*, commonly known as Indian ginseng (Family: Solanaceae) and *Morinda citrifolia*, commonly known as Indian mulberry (Family: Rubiaceae) have been shortlisted for the study and collected from the Department of Horticulture, Division of Aromatic and Medicinal Plants, University of Agricultural Sciences (UAS), GKVK, Bangalore. Leaves, stem, roots and flowers of the respective plant parts, as applicable, were studied for their endophytic population and lovastatin producing capacity.

Samples identification

Authentication of the plant was done by the Department of Botany, University of Agricultural Sciences (UAS), Bangalore.

Isolation of endophytic fungi

Leaves, stems, roots and flowers (as applicable) of the respective plants were surface sterilized using 0.1% tween-80 (60 minutes), 70% v/v ethanol (15 seconds) and sodium hypochlorite (15 minutes), finally washed with distilled water and blot dried. The sterilized plant parts were cut into 0.5 cm x 0.5 cm and placed on streptomycin (150 mg/L) supplemented Potato Dextrose Agar (PDA) to prevent bacterial contamination and incubated at 25 °C for 2 weeks[24]. After the incubation period, hyphal tips associated with the plant tissue were transferred onto fresh PDA plates in pure culture followed by sub-culturing once in every four weeks.

Identification and screening of endophytic fungi

Morphological identification of all isolates was carried out using lactophenol cotton blue stain. Each of the fungal isolates was subjected to submerged fermentation (SmF) using soybean meal medium consisting of sucrose (50 g/L), soybean meal (20 g/L), K_2HPO_4 (1 g/L), $NaNO_3$ (1 g/L), $MgSO_4.7H_2O$ (0.5 g/L); pH 6.5. The flasks were incubated at 28 °C for 7 days in a rotary shaker at 100 rpm[25]. Two rounds of screening were done at this step to ensure the consistency of endophytes and check the level of gene attenuation at each level of screening.

Lovastatin extraction

Equal quantity of ethyl acetate was added to the fermented material, whose pH was adjusted to 2 using

1N HCl and kept in a rotary shaker for 2 hours at 100 rpm for the lovastatin to pass into the organic phase. The organic phase is separated and allowed to dry. The dried residue is reconstituted in 1 mL ethanol and stored at 4 °C till further analysis[26].

Qualitative analysis

The presence of lovastatin in the fermented material was confirmed by Thin Layer Chromatography (TLC) using Toluene and ethanol as a solvent system in the ratio 80:20[25]. The samples were run against standard lovastatin and the R_f values were calculated. The antifungal property of lovastatin was demonstrated by *Saccharomyces cerevisiae* bioassay on Yeast extract Peptone Dextrose Agar (YPDA) plates, ethanol being the control. After 18 hours of incubation, the zone of inhibition was measured[27].

Quantification of lovastatin

Lovastatin was quantified by adding 1 mL of alkaline hydroxylamine, 5 mL of ferric perchlorate and 1 mL of 2N HCl to 0.5 mL of fungal extract and making up the volume of the system to 10 mL with ethanol[28]. After incubating the system at room temperature for 25 minutes, the optical density of the resultant dark purple-coloured solution was read at 513 nm.

All the experiments were carried out in triplicates and data was expressed as mean±standard error.

Results and Discussion

A total of 98 fungal isolates were obtained from the surface-sterilized plant parts (Table 1). All the plant parts studied were found to be inhabited by at least one species of endophytes supporting Arnold *et al.*[29]. The isolates were stained with lactophenol cotton blue and observed under the light microscope to be *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Trichoderma* sp., *Mucor* sp., *Rhizopus* sp., and *Candida* sp. *Aspergillus* sp. deserves a mention as the most commonly isolated fungi from almost all the leaf tissues of the medicinal plants under study. Raghunath *et al.*[30] reported *Aspergillus* sp.,

Table 1 — Lovastatin producing endophytic fungi isolated from leaf, stem, root and flower (as applicable) of the plants

S. No	Plant	Plant part	Organisms	No. of Lovastatin producing strains
1	*Ocimum tenuiflorum*	Leaf	*Aspergillus* sp.	5
		Stem	*Aspergillus* sp.	4
		Root	*Penicillium* sp.	3
2	*Emblica officinalis*	Leaf	*Aspergillus* sp.	5
		Stem	*Fusarium* sp.	1
		Root	*Aspergillus* sp.	3
3	*Moringa oleifera*	Leaf	*Trichoderma* sp.	2
		Stem	*Aspergillus* sp.	3
		Root	*Aspergillus* sp., *Penicillium* sp.	6
4	*Bacopa monnieri*	Leaf	*Aspergillus* sp., *Trichoderma* sp.	3
		Stem	*Aspergillus* sp.	4
		Root	*Aspergillus* sp.	3
5	*Murraya koenigii*	Leaf	*Aspergillus* sp.	4
		Stem	*Aspergillus* sp., *Mucor* sp.	3
		Root	*Penicillium* sp.	3
6	*Azadirachta indica*	Leaf	*Aspergillus* sp.	3
		Stem	*Aspergillus* sp., *Mucor* sp.	2
		Root	*Aspergillus* sp., *Rhizopus*	2
7	*Hibiscus rosa-sinensis*	Leaf	*Aspergillus* sp., *Candida* sp.	5
		Stem	*Aspergillus* sp.	3
		Root	*Aspergillus* sp.	4
		Flower	*Aspergillus* sp.	3
8	*Catharanthus roseus*	Leaf	*Aspergillus* sp., *Fusarium* sp.	2
		Stem	*Aspergillus* sp.	2
		Root	*Penicillium* sp.	3
		Flower	*Aspergillus* sp.	3
9	*Withania somnifera*	Leaf	*Aspergillus* sp.	2
		Stem	*Aspergillus* sp.	3
		Root	*Aspergillus* sp,. *Penicillium* sp.	2
10	*Morinda citrifolia*	Leaf	*Aspergillus* sp.	3
		Stem	*Aspergillus* sp.	2
		Root	*Penicillium* sp.	2

Penicillium sp., *Alternaria* sp., *Fusarium* sp. and *Mucor* sp. as the most frequently isolated genera from endophytic *Taxus baccata*.

In the current study, all the isolates except those belonging to *Mucor* sp. and *Rhizopus* sp. were found to be lovastatin producers. The range of yield from lovastatin producers in the first round of submerged fermentation was recorded between 5 and 71.5 mg/L.

Lovastatin production was first reported from *Penicillium* sp.[31] Later several genera such as *Monascus* sp., *Aspergillus* sp., *Paecilomyces* sp., *Trichoderma* sp., *Phoma* sp., *Pythium* sp., *Pleurotus* sp. and *Fusarium* sp., have been reported to produce lovastatin[32-35]. Samiee *et al.*[36] documented lovastatin production from various species belonging to the genus *Aspergillus* such as *Aspergillus terreus, A. parasiticus, A. fischeri, A. flavus* and *A. umbrosus*. In studies conducted by Osman *et al.*[37], soil fungi such as *Aspergillus* sp., *Penicillium* sp., *Biospora* sp., *Cylindrocarpon* sp. and *Trichoderma* sp. were reported as lovastatin producers. Jaivel and Marimuthu[38] reported lovastatin production from soil isolates such as *Aspergillus* sp., *Monascus* sp., *Penicillium* sp., *Pleurotus* sp. and *Trichoderma* sp. In all the studies cited above, *A. terreus* was found to be the best producer of lovastatin. In contrast to what was found in this study, *Rhizopus oryzae* was reported to produce lovastatin with a considerable yield in a study conducted by Immanuel *et al.*[39] but from soil sources.

Lately, plants are being screened for the isolation of potent lovastatin producing endophytic fungi that have a yield on par with well exploited and established soil fungi. Contradicting the findings of Praveen *et al.*[7] that declare endophytes as poor lovastatin producers, there are reports on lovastatin production from endophytic *A. niger* PN2 from the leaves of *Taxus baccata*[30] and endophytic *Phomopsis vexans* from the leaves of the medicinal plant *Solanum xanthocarpum*[8]. Of late, an endophytic *Aspergillus luchuensis* MERV10 isolated from marine mangrove was shown to exhibit highest lovastatin productivity[40]. Majority of the studies carried out concludes *A. terreus* as the most promising producer of lovastatin and hence it is being used for the commercial production of lovastatin at industrial level.

Since endophytes are hindered by a phenomenon called gene attenuation on repeated subculturing[41], the most potent isolates from each level were further screened up to three levels to check their potency and consistency in terms of lovastatin production. It was found that only three isolates from the leaves of *Hibiscus rosa-sinensis* maintained the same yield through all rounds of screening. One among the three isolates (designated and mentioned further as HL1) identified to be *Candida sp.* is being reported for the first time as lovastatin producer in the current study with a considerably high yield of 40 mg/L. The other two potent isolates (designated and mentioned further as HL4 and HL5) were identified to be *A. terreus*, with a yield 21.5 and 18 mg/L respectively. The consistency in lovastatin production from these fungi across all levels of screening could be attributed to the fact that the biosynthesis of the metabolite is inherently encoded in the genomes of these endophytic fungi[42]. The methanolic leaf extracts of *Hibiscus rosa-sinensis* were shown to have lipid-lowering ability[43]. However, the study was focused on the phytochemical analysis of the leaf extracts and does not explain if the lipid-lowering property is attributed to the presence of lovastatin producing endophytic fungi.

TLC results confirm the presence of lovastatin in the fungal extract through R_f values which are 0.69 for the standard lovastatin and 0.70, 0.71, and 0.71 for HL1, HL4, and HL5 respectively. The bioassay against *S. cerevisiae* validates antifungal activity of the fungal extracts by the formation of a zone of inhibition around the wells loaded with the sample. The diameter of the zones of inhibition formed by the extracts from HL1, HL4 and HL5 was 1.1, 1.2, and 1.1 cm respectively whereas that formed by standard lovastatin was 1.1 cm.

Conclusion

This study caters to the need for exploiting various ecological niches for the isolation of potent lovastatin producing fungi. To the best of our knowledge, this is the first report on lovastatin production from endophytic yeast belonging to *Candida* sp. with an appreciably high yield which is on par with *A. terreus* from other sources. Also, lovastatin production from *A. terreus* with substantial yield from sources other than soil is being reported for the first time. Further, the production can be scaled up cost-effectively through solid-state fermentation using various inexpensive and widely available agro and food wastes.

Conflict of interest

The authors declare that they have no conflict of

interests

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