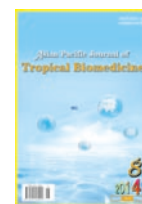


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Polyketide and benzopyran compounds of an endophytic fungus isolated from *Cinnamomum mollissimum*: biological activity and structureCarolina Santiago¹, Lin Sun², Murray Herbert Gibson Munro², Jacinta Santhanam^{1*}¹Biomedical Science Programme, School of Diagnostic and Applied Health Sciences, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia²Department of Chemistry, University of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand

PEER REVIEW

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Comments

This study has studied the bioactivity of three compounds isolated from *Phoma* sp. fungus. The findings are well represented which indicated endophytic fungi may have a significance meaning in the drug discover.

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ABSTRACT

Objective: To study bioactivity and compounds produced by an endophytic *Phoma* sp. fungus isolated from the medicinal plant *Cinnamomum mollissimum*.

Methods: Compounds produced by the fungus were extracted from fungal broth culture with ethyl acetate. This was followed by bioactivity profiling of the crude extract fractions obtained via high performance liquid chromatography. The fractions were tested for cytotoxicity to P388 murine leukemic cells and antimicrobial activity against bacteria and pathogenic fungi. Compounds purified from active fractions which showed antibacterial, antifungal and cytotoxic activities were identified using capillary nuclear magnetic resonance analysis, mass spectrometry and admission to AntiMarin database.

Results: Three known compounds, namely 4-hydroxymellein, 4,8-dihydroxy-6-methoxy-3-methyl-3,4-dihydro-1H-isochromen-1-one and 1-(2,6-dihydroxyphenyl) ethanone, were isolated from the fungus. The polyketide compound 4-hydroxymellein showed high inhibitory activity against P388 murine leukemic cells (94.6%) and the bacteria *Bacillus subtilis* (97.3%). Meanwhile, 4,8-dihydroxy-6-methoxy-3-methyl-3,4-dihydro-1H-isochromen-1-one, a benzopyran compound, demonstrated moderate inhibitory activity against P388 murine leukemic cells (48.8%) and the fungus *Aspergillus niger* (56.1%). The second polyketide compound, 1 (2,6-dihydroxyphenyl) ethanone was inactive against the tested targets.

Conclusions: These findings demonstrate the potential of endophytes as producers of pharmacologically important compounds, including polyketides which are major secondary metabolites in fungi.

KEYWORDS

Endophytic fungi, HPLC bioactivity profiling, *Cinnamomum mollissimum*, Antimicrobial

1. Introduction

In 1996, the discovery of taxol, an anticancer compound (originally isolated from the plant *Taxus brevifolia*) from an endophytic fungal species *Pestalotiopsis microspora*, highlighted endophytic fungi as an alternate source for drug discovery[1]. Following this discovery, many studies reported

the enormous potential of natural products from endophytes which exhibited antimicrobial, antiviral, antiparasitic and antitumor properties[2]. Endophytic fungi are described as fungi which live within plant tissue with no evidence of symptoms to the host plant. Some of these endophytes provide protection to their host plant from tissue invading pathogens by producing compounds that are involved the

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host plant's defense mechanism^[3–5]. The fungi have been shown to produce similar compounds as their host plant^[6,7]. This provided the rationale to investigate the bioactivity of compounds produced by endophytes, especially those isolated from medicinal plants.

Plants of *Cinnamomum* species (sp.) are known to possess antimicrobial activities and are widely applied in herbal therapy in treating colds, sinusitis, bronchitis and fungal infection^[8,9]. A wide range of biological activities ranging from antiseptic, antitumor to antifungal were reported to be found in constituents and essential oils of *Cinnamomum* sp. plants^[10–12]. In this research, an endophytic fungus isolated from *Cinnamomum mollissimum* (*C. mollissimum*) was studied. The fungal isolate CB 007 [water agar (WA)], a *Phoma* sp., was previously investigated, and found to produce 5-hydroxyramulosin, a compound that was active against *Aspergillus niger* (*A. niger*) (IC₅₀ 1.56 µg/mL) and cytotoxic against murine leukemia cells (IC₅₀ 2.10 µg/mL)^[13]. In this study, we report the isolation and biological activity of other compounds produced by CB 007 (WA).

2. Materials and methods

2.1. Sampling of *C. mollissimum*

Plant parts of *C. mollissimum* were obtained from Universiti Kebangsaan Malaysia Forest Reserve, in Bangi, Selangor, Malaysia. Several twig samples were sampled from a young tree approximately 1.3 m tall. Samples were stored at 4 °C until further processing. The plant specimen voucher (No. 955) was deposited in the herbarium of Universiti Kebangsaan Malaysia and identified by university's botanist.

2.2. Isolation of endophytes

Plant samples were processed according to methods described by Jayanthi *et al.* 2011^[14] and incubated on potato dextrose agar and WA at 27 °C. Emergence of hyphal tips was periodically checked for 3–14 d. The individual hyphal tips were removed and cultured on potato dextrose agar at 27 °C and culture purity was monitored regularly. One of the fungal isolates, CB 007 (WA) was selected for further experimentation, based on the preliminary screening results^[13].

2.3. Fermentation and extraction of fungal metabolites

The fungal isolate was cultured in 1000 mL Erlenmeyer flask containing 200 mL of potato dextrose broth, shaken at 140 r/min, 27 °C for 14 d. Mycelia were removed via filtration and the culture broth was extracted with an equal amount of ethyl acetate overnight. The filtrate was concentrated by removing the solvents under reduced pressure at 35–

40 °C with a rotary evaporator (Büchi, New Castle, USA). The concentrated crude extract was ensured to be dry and stored at 4 °C.

2.4. High performance liquid chromatography (HPLC) analysis

An aliquot of the crude extract (250 µg) was analyzed by reverse phase C₁₈ HPLC using a gradient solvent system comprising acetonitrile and ultra-pure water as described in methods by Santiago *et al.* 2012^[13]. HPLC was performed on a Dionex (Sunnyvale, USA) system equipped with an ISCO Foxy Jr. sample collector using a reversed-phase analytical column (Phenomenex Prodigy C₁₈, 4.6 mm×250 mm, 5 µm) with photodiode array and an Alltech evaporative light scattering detection (ELSD) (Grace, Deerfield, USA).

2.5. Bioactivity profiling–cytotoxicity assay

Fractions from crude extract (88 fractions×200 µL) were collected in a microtiter plate from HPLC analysis. Daughter plates were made by transferring 50 µL from the original microtiter plate to another plate for analysis of cytotoxicity against P388 murine leukemic cells. The solvent was evaporated prior to the assay by using a centrifugal evaporator. Medium used for the cytotoxicity assay was β-methoxyethoxymethyl, fetal calf serum (10%), penicillin (266 µg/mL), streptomycin (132 µg/mL), L-glutamine (0.002 mol/L), sodium bicarbonate (2.2 g/L), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (0.0074 mol/L). The plate was incubated 36 °C for 3 d. To obtain the result, 20 µL of thiazolyl blue tetrazolium solution (3.8 mg/mL in phosphate buffered saline) was added to every well and the plate was incubated for 4 h at 36 °C. After incubation, hydrochloric acid in isopropanol (170 µL, 0.08 mol/L) was used to dissolve the formazon product. Cell viability was determined by measuring the absorbance of every well at 540 nm. The absorbance of cell free control and the analyte free cell control was taken as 0% and 100% growth reference, respectively. Cisplatin was used as reference positive control in this experiment.

2.6. Bioactivity profiling–antimicrobial assay

The assay was done according to methods described in Santiago *et al.* 2012^[13]. A second microtiter plate containing fractions (88 fractions×200 µL) was obtained from HPLC analysis and assayed directly after evaporating the solvent with a centrifugal evaporator. Each well was added with 10 µL of 5% v/v methanol in water and 50 µL growth media. Media used for antifungal assay was RPMI-1640 and for antibacterial assay was Mueller Hinton broth. This was followed by addition of 40 µL of suspension of test organisms. The inoculum size of test organisms used was 2.5×10³ CFU/mL for the pathogenic fungi *A. niger* and *A.*

fumigatus and 5×10^5 CFU/mL for *Bacillus subtilis* (*B. subtilis*) bacteria. Plates were incubated at 27 °C, 48 h for *A. niger* and *A. fumigatus*; 37 °C, 24 h for *B. subtilis*. After incubation, an tetrazolium solution (20 µL, 5 mg/mL) was added to all wells and plates were further incubated (27 °C, 4 h). Then, 100 µL of dimethyl sulfoxide was added into every well to dissolve the formazon product. Cell viability percentage was determined by measuring the absorbance of every well at 540 nm and subtracting the absorbance of cell free control. The absorbance of cell free control (solvent solution+medium) and the analyte free cell control (solvent solution+test organisms in medium) was taken as 0% and 100% growth reference, respectively. Cell growth inhibition was calculated as $100\% - \text{cell viability}\%$. The antibiotic gentamycin and antifungal amphotericin B used as positive controls in this experiment.

2.7. Isolation of compounds

The crude extract was subjected to HPLC by injecting 250 µg of extract onto a C₁₈ column (250 mm×4.6 mm, 5 µm, Phenomenex Luna) using the same gradient solvent system as before for HPLC analysis. The collection of the bioactive fractions was scaled up to 10 times, to collect the pure compounds.

2.8. Determination of compounds

The pure compounds were subjected to nuclear magnetic resonance (NMR) and mass spectrometry experiments were for identification. The NMR experiments was recorded on

a Varian INOVA 500 spectrometer (Varian, Palo Alto, USA) at 23 °C, operating at 500 MHz at 23 °C, using a capillary probe. High resolution electron impact mass spectra were obtained on a liquid chromatography time–time of flight mass spectrometer (Micromass, Greater Manchester, UK). Structural elucidation of compounds process was aided by access to AntiMarin database.

3. Results

In bioactivity profiling, HPLC fractions were assayed on a microtiter plate for cytotoxicity and antimicrobial activity. A line graph was plotted to quantitatively indicate the inhibitory activity of HPLC fractions. These fractions correspond directly to collection time over 40 min from fraction 1 being the first collected fraction to fraction 88 the last collected fraction. Figure 1 is showing bioactivity profile for first 36 HPLC fractions obtained from CB 007 (WA). The remaining 46 fractions which eluted later (after fraction 36) did not exhibit any biological activity; hence the results were not included in Figure 1. An active region shown on the graph was interpreted as where most of the biological activity was found. The active region for CB 007 (WA) extract was from fraction 28 to 34 in which it was observed inhibitory activities against P388 murine leukemic cells, *B. subtilis* and *A. niger* (Figure 1). Several few fractions which eluted earlier (fraction 3 to 13) indicated the potent antifungal activity against *A. fumigatus*.

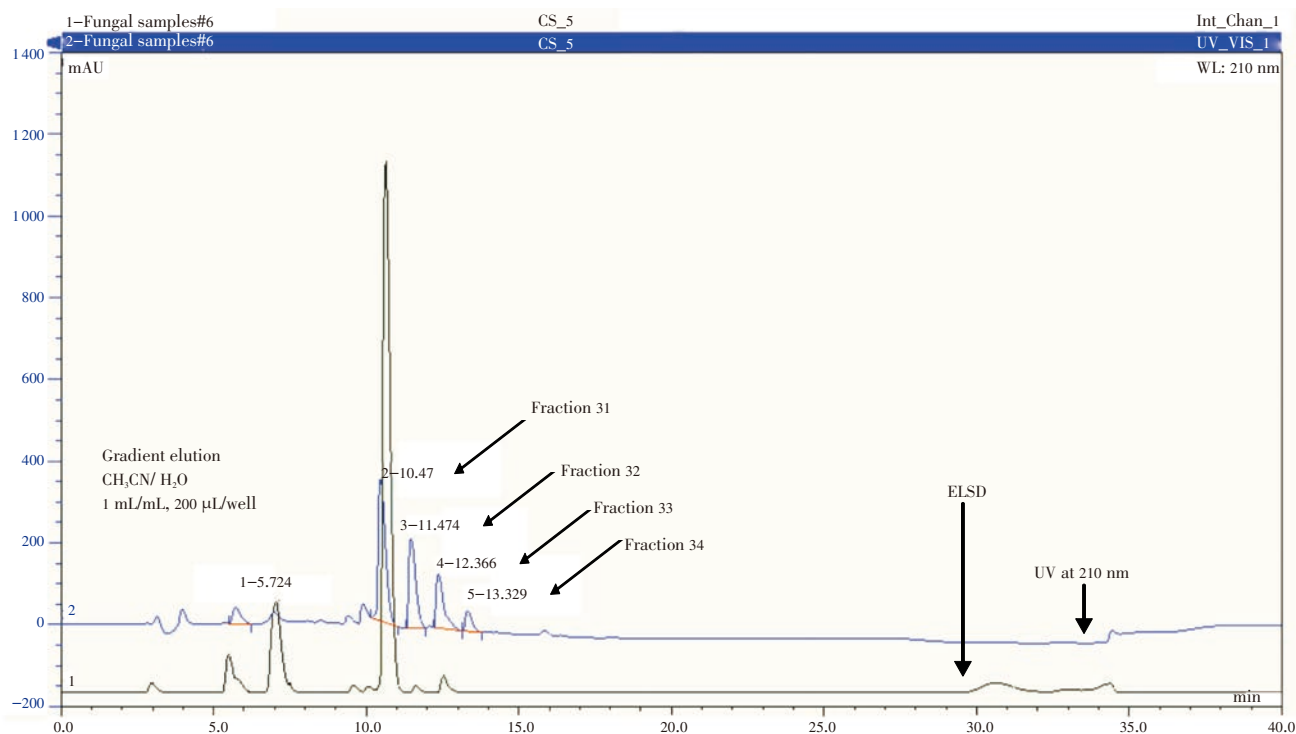


Figure 2. HPLC chromatogram with ELSD and UV detection, of 250 µg fungal endophyte CB 007 (WA) extract, showing major chromatograph peaks present in the biologically active region consisting fraction 31, 32, 33 and 34 which correlated to collection time between 10 to 14 min.

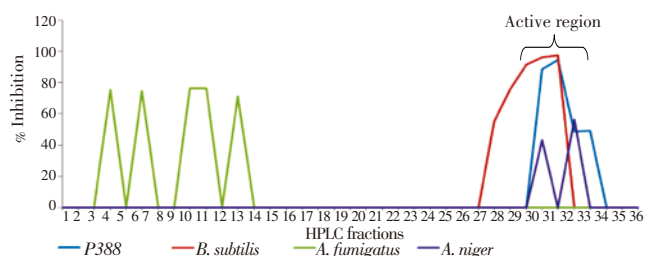


Figure 1. Bioactivity profile of HPLC fractions (fraction 1 to 36) from the fungal endophyte CB 007(WA) extract showing inhibition of P388 murine leukemic cells, *B. subtilis*, and *A. niger* in active region.

An active region is interpreted as region with most bioactivity observed as indicated by the marked area in the graph.

The bioactivity profiling led to identification of active region which directly corresponded to a specific time region between 10 to 14 min in the HPLC chromatogram (Figure 2). Four major peaks were present in the chromatogram in this time frame and each fraction that correlated to the peaks were collected and purified. This led to isolation of 4-hydroxymellein (A), 4,8-dihydroxy-6-methoxy-3-methyl-3,4-dihydro-1H-isochromen-1-one (B) and 1-(2,6-dihydroxyphenyl) ethanone (C) as depicted in Figure 3. The compound A, B and C eluted after 11.5 (fraction 32), 12.4 (fraction 33) and 13.2 (fraction 34) min, respectively (Figure 2). The compound 5-hydroxyramulosin has been reported previously^[13,15] eluted after 10.47 min (fraction 31). The spectral data for compounds A, B and C is as following:

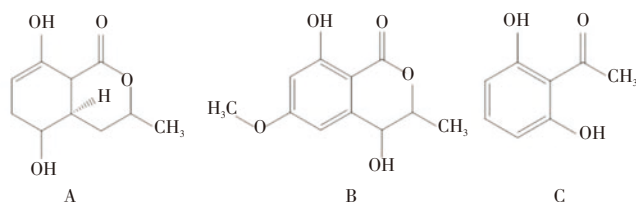


Figure 3. Structure of 4-hydroxymellein (A), 4,8-dihydroxy-6-methoxy-3-methyl-3,4-dihydro-1H-isochromen-1-one (B) and 1-(2,6-dihydroxyphenyl) ethanone (C).

4-Hydroxymellein (A): white solids; UV (MeOH) λ_{\max} 210, 245, 314 nm. HRESIMS m/z 193.0685 $[M+H]^+$. 1H and ^{13}C NMR data were consistent to previously reported literature^[16,17]; Formula $C_{10}H_{10}O_4$.

4,8-Dihydroxy-6-methoxy-3-methyl-3,4-dihydro-1H-isochromen-1-one (B): white solids; UV (MeOH) λ_{\max} 215, 266, 301 nm. HRESIMS m/z 255.0744 $[M+H]^+$. Natural compound for stock screening; Formula $C_{11}H_{12}O_5$.

1-(2,6-dihydroxyphenyl) ethanone (C): white solids; UV (MeOH) λ_{\max} 210, 243, 310 nm. HRESIMS m/z 142.0443 $[M]$. 1H and ^{13}C NMR data were consistent to previously reported literature^[18]; Formula $C_8H_8O_3$.

The biological activity of bioactive HPLC fractions from CB 007 (WA) and compounds isolated from them is summarized in Table 1. Compound A has inhibitory activity against P388 murine leukemic cells and *B. subtilis*. Compound B was inhibiting P388 murine leukemic cells and *A. niger*. However, compound C was inactive in all tested assays.

Table 1

Biological activity and major compounds isolated from HPLC fractions of the fungal endophyte CB007 (WA) extract.

Fractions	Biological activity (% inhibition)			Isolated compound
	P388	<i>B. subtilis</i>	<i>A. niger</i>	
32	94.6	97.3	^a –	A
33	48.8	–	56.1	B
34	–	–	–	C
^b Positive control	92.6	97.0	96.6	

^ano activity observed; ^bPositive controls used in the assay; cisplatin for P388 murine leukemic cell testing, gentamycin for antibacterial testing and amphotericin B for antifungal testing.

4. Discussion

Endophytes are known to produce pharmacologically active compounds. The strategy in this research was to explore endophytic fungi from a medicinal plant. *Cinnamomum* plants are widely utilized as natural remedies to cure many diseases because of their healing properties. In herbal therapeutics, essential oils from the leaf, inner bark and stems of the plant are used as remedies^[10]. Biologically active compounds from endophytes of *Cinnamomum* sp. have been studied previously but there is no reported study to date on endophytes from *C. mollissimum*.

The endophyte CB 007 (WA) showed encouraging antimicrobial and cytotoxic activity in a screening assay^[13]. Therefore, the research was undertaken to identify the compounds that gave rise to the observed activity. Metabolites from the isolate were extracted and analysed. This eventually led to the isolation of three known compounds; 4-hydroxymellein, 4,8-dihydroxy-6-methoxy-3-methyl-3,4-dihydro-1H-isochromen-1-one and 1-(2,6-dihydroxyphenyl) ethanone.

The compounds 4-hydroxymellein and 1-(2,6-dihydroxyphenyl) ethanone are polyketides that are synthesized by the polyketide synthase pathways. 4-hydroxymellein was first isolated from a fungus, *Cercospora taiwanensis*. Fungus belonging to this genus is known to be pathogenic to crops such as sugarbeets and soyabeans^[16]. A recent research also reported the isolation of 4-hydroxymellein from an endophytic fungus of *Penicillium* sp., which exhibited active anti-fungal activities^[19]. Meanwhile, the compound 1-(2,6-dihydroxyphenyl) ethanone was firstly discovered from *Daldinia concentrica*, a fungus belonging to Ascomycota division^[18]. No biological activities has been reported for this compound. Polyketides compounds such as the above mentioned compounds are results of condensation of activated primary metabolites (acetyl-CoA and malonyl-CoA) to form β -ketoacetyl polymers which are linked to the enzyme by thioester bonds. They are structurally and functionally related to fatty acid synthases^[20] and are identified by a common biosynthetic origin of carbon atoms derived from small carboxylic

acids^[21]. Polyketides are frequently found as fungal metabolites and they are an important class of compounds in natural product drug discovery due to their structural diversity. They possess remarkable biological activity as antibiotic, antifungal, anticancer, antiparasitic, and immunosuppressant^[22]. Examples of polyketides that have been commercialized include amphotericin B (antifungal), lovastatin (anti-cholesterol), rapamycin (immunosuppressant) and erythromycin B (antibiotic). As such, the two polyketides compounds A and C can be potentially developed into drug in medicine industry.

Compounds of mellein derivatives have been reported to display antimicrobial and cytotoxic activity which is supported by the observation in this study^[23]. 4-hydroxymellein demonstrated inhibitory activity against *B. subtilis* and P388 murine leukemic cells (97.3% and 94.6% respectively) which has not been reported before. However, 1-(2,6-dihydroxyphenyl) ethanone was inactive for the tested activity. Assays conducted in this research were limited to evaluate antibacterial, antifungal and leukemia cytotoxic activity. As such, other *in vitro* assays such as antitumor or anti-parasitic assays can be employed to fully examine the potential of 1-(2,6-dihydroxyphenyl) ethanone.

The compound 4,8-dihydroxy-6-methoxy-3-methyl-3,4-dihydro-1H-isochromen-1-one showed moderate inhibitory activity against P388 murine leukemic cells (48.8%) and *A. niger* (56.1%) and no other biological activities were reported for this particular compound found from the literature review. This compound belongs to the benzopyran group which is an organic compound that is produced by fusion of benzene ring to heterocyclic pyran ring. Since benzopyrans are associated with polyketide synthesis this compound may have arisen from the same carbon framework as 4-hydroxymellein and 1-(2,6-dihydroxyphenyl) ethanone via polyketide synthase action. Among natural products, benzopyrans were found to be the core ring structures of flavonoids, isoflavanoids and isocoumarins^[24]. Benzopyran derivatives possess numerous pharmacological properties as diuretic, analgesic, myorelaxant, and hypoglycaemic^[25–27]. Besides this, benzopyrans are potential intermediates in the synthesis of steroid analogs, or function as building blocks in the production of pterocarpan and isoflavones that have strong fungicidal activity^[28]. Hence, the fungicidal activity of compound B observed in this study is attributed to its structural and functional similarities to pterocarpan and isoflavones.

Although, this study reports three known compounds, new biological activities have been identified in these compounds. In addition, the activity of the compounds is specific for either bacteria or fungi alone unlike toxic compounds which destroy all cell types. Therefore, further studies are needed to exploit the full potential of these compounds as antifungal or antibacterial agent. We conclude that endophytic fungi from medicinal plants is a significant resource for drug discovery.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

The present investigation demonstrates the bioactivity of the three known compounds isolated from *Phoma* sp. in *C. mollissimum*. This may lead to further research and explore on the potential of endophytic fungal (*Phoma* sp.) and their active compounds.

Research frontiers

The author found three active compounds from *Phoma* sp. in *C. mollissimum* and its biological activity. *Phoma* sp. extract contained 4-Hydroxymellein, 4,8-Dihydroxy-6-methoxy-3,4-dihydro-1H-isochromen-1-one and 1-(2,6-dihydroxyphenyl) ethanone. These compounds exhibited inhibition against P388, *B. subtilis* and *A. niger*.

Related reports

So far, many research have been done on the isolation of endophytic fungi from medicinal plants. The present work revealed on the three known compounds isolated from *Phoma* sp. with their bioactivity.

Innovations and breakthroughs

Several studies were carried out on the isolation of endophytic fungi from *C. mollissimum*. In the present study, authors have worked on the isolation of three active compounds from *Phoma* sp. endophytic fungi.

Applications

The present investigation demonstrates the bioactivity of the three known compounds isolated from *Phoma* sp. in *C. mollissimum*. This may lead to further research and explore on the potential of endophytic fungal (*Phoma* sp.) and their active compounds.

Peer review

This study has studied the bioactivity of three compounds

isolated from *Phoma* sp. fungi. The findings are well represented which indicated endophytic fungi may have a significance meaning in the drug discover.

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