Ann Microbiol (2017) 67:255–261
DOI 10.1007/s13213-017-1256-4

ORIGINAL ARTICLE

Isolation and characterization of trichalasin-producing endophytic fungus from *Taxus baccata*

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Received: 20 August 2016 / Accepted: 18 January 2017 / Published online: 9 February 2017

Abstract An endophytic fungus (strain T1) isolated from *Taxus baccata* was studied for the production of metabolites with anticancer and antioxidant activities. This fungus was identified as *Diaporthe* sp. based on rDNA-internal transcribed spacer (ITS) sequence analysis. The crude extract showed cytotoxic activity against MCF-7 and HeLa cancer cell lines, with IC_{50} (concentration inhibiting 50% of growth rate) values of 1058 ± 44 and 1257 ± 80 μg ml^{-1}, respectively. The scavenging activity of fungal extract increased significantly with increasing concentration [IC_{50} (concentration required to scavenge 50% of free radicals) 482 ± 9 μg ml^{-1}]. Ultra-high-performance liquid chromatography-quadrupole-time of flight analysis revealed the presence of three trichalasins (trichalasin E, F and H) in the crude extract of T1 which are known to have antitumour and antioxidant activities. These results suggest that *Diaporthe* sp. has the potential to be used for therapeutic purposes because of its antiproliferative and antioxidant potential and also for the production of cytochalasins.

Keywords Endophytic fungi · *Diaporthe* · Cytochalasins · Trichalasins · Antioxidant activity

Electronic supplementary material The online version of this article (doi:10.1007/s13213-017-1256-4) contains supplementary material, which is available to authorized users.

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Introduction

Endophytes are ubiquitous organisms which reside in the tissues of plants without causing apparent symptoms of disease (Bacon and White 2000). The majority of endophytes are fungi (Strobel and Daisy 2003), and they are present in the intercellular spaces of various plant parts, such as the leaf and root (Corrêa et al. 2014). Endophytes reduce damage to their host plants by producing many secondary metabolites (Cabezas et al. 2012). They are also able to inhibit infection and proliferation of pathogens in the host plant directly or indirectly by inducing resistance responses intrinsic to the host defence (Eaton et al. 2011). Currently, endophytic fungal research is focused on the ability of these fungi to produce and accumulate secondary metabolites. Several of these compounds have biological activities of interest for application in environmental, agriculture, pharmaceutical and healthcare and food industries (Suryanarayanan et al. 2009; Kharwar et al. 2011; Deshmukh et al. 2015).

Diverse classes of chemical substances, such as steroids, xanthones, phenols, isocoumarines, perylene derivatives, quinones, furandiones, terpenoids, depsipeptides and cytochalasins, have been isolated from endophytic fungi (Schulz and Boyle 2005). Endophytes produce a significantly higher number of novel chemical structures than soil fungi, indicating that endophytes are a novel source of bioactive secondary metabolites (Nisa et al. 2015). In this context, the secondary metabolites produced by endophytes associated with medicinal plants are important as they could be exploited for the treatment of many diseases (Tejesvi et al. 2007). Several studies have shown that endophytes produce secondary metabolites of their host plants, thus raising the prospect of using them as alternative sources of these metabolites (Priti et al. 2009).

Endophytic fungi of the genus *Diaporthe* have been isolated from a wide variety of plant hosts from both temperate and

tropical regions (Dos Santos et al. 2016). *Diaporthe* is one of the most abundant genera of endophytic fungi and has been reported to produce various compounds of biotechnological interest (Silva et al. 2005; Pornpakakul et al. 2007; Rukachaisirikul et al. 2008). Cytochalasins are a group of cytotoxic fungal metabolites showing a wide range of biological activities, such as anticancer, antimicrobial, antiparasitic, phytotoxic activities, among others (Scherlach et al. 2010; Chen et al. 2014). These metabolites have been isolated from many fungal species, including *Helminthosporium* sp., *Phoma* sp., *Xylaria* sp., *Hypoxylon* sp. and *Rhinocladiella* sp. (Scherlach et al. 2010).

In the investigation reported here, we isolated an endophytic fungus from the bark of the *Taxus baccata* L. subsp. *wallichiana* (Zucc.) Pilger (Himalayan Yew) and subsequently identified it as *Diaporthe* sp. strain T1 based on its internal transcribed spacer (ITS) regions of rDNA. The biological activities, such as anticancer and antioxidant activities, of ethyl acetate extracts of its culture filtrate were studied using various bioassays. These extracts were also analysed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) to identify some key compounds belonging to the cytochalasin group of fungal metabolites which could be responsible for these activities.

Material and methods

Isolation and identification of endophytic fungus strain T1

Endophytic fungus T1 was isolated from the bark samples of *T. baccata* L. subsp. *wallichiana* (Zucc.) Pilger collected from Bhadrewah (Doda district, India) according to the method described by Garyali et al. (2013). The fungal culture was maintained on potato dextrose agar (HiMedia Laboratories Ltd. Mumbai, India) medium. Molecular characterization was performed by ITS sequence analysis. Specifically, mycelia were harvested from actively growing colonies and ground in liquid nitrogen. Genomic DNA was extracted from the finely ground fungal material by the CTAB method (Zhang et al. 2008). The quality and quantity of DNA was checked with Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and samples were stored at −20 °C until use. The ITS region of nuclear ribosomal DNA was amplified with the universal primers ITS1 and ITS4 (White et al. 1990) in a thermal cycler (Applied Biosystems, Foster City, CA). The thermal cycling conditions applied for the ITS region consisted of an initial denaturation for 5 min at 94 °C followed by 34 cycles of 1 min at 94 °C, 1 min at 50 °C and 1.5 min at 72 °C and a final extension of 7 min at 72 °C. PCR products were purified using QIAquick spin columns (Qiagen, Hilden, Germany) following the manufacturer's instructions and then sequenced. The ITS sequence obtained from this isolate has been deposited in the GenBank under accession number KX355165.

The ITS sequence of T1 was compared to those available in the GenBank database using the BLASTN algorithm. Alignment of the sequences was constructed using MAFFT version 7.0 (Katoh and Standley 2013) and edited with BioEdit version 5.0.6 (Hall 1999). Phylogenetic analysis on the resulting alignment was performed using Bayesian Inference. A Bayesian analysis was implemented in MrBayes v.3.2.2 with two parallel runs, each consisting of four incrementally heated Monte Carlo Markov Chains. The analysis was run using Metropolis-coupled a Markov Chain Monte Carlo search algorithm over 1,000,000 generations, and the convergence of Bayesian analysis was observed by examination of the standard deviation of split frequencies of <0.01. Trees were sampled every 100th generations resulting in total of 10,000 trees. The first 2500 trees, representing the burn-in phase of the analysis, were discarded, and the remaining 7500 trees were used to calculate posterior probabilities from the 50% majority rule consensus trees.

Fungal extract preparation

Mycelial discs (diameter 5.0 mm) of actively growing culture (7 days old) were inoculated into potato dextrose broth (500 ml) and incubated at 25 ± 2 °C for 21 days in the dark as a static culture. The cultures were harvested by filtration through four layers of cheesecloth to remove the mycelia, and the mycelial biomass thus obtained was then dried overnight (35–40 °C) and extracted with ethyl acetate for 12 h. The culture broth was also extracted three times with an equal volume of ethyl acetate; the ethyl acetate fractions were then pooled and dried in vacuo at 35 °C. Part of the residue was dissolved in dimethyl sulfoxide and used for the bioassays. The other part of the residue was dissolved in methanol and partitioned three times with an equal volume of dichloromethane); the dichloromethane fractions were pooled and dried in vacuo. The residue was used for the LC–two-dimension-MS [ultra-high-performance LC-quadrupole-time of flight analysis (UHPLC-QTOF-MS/MS)] to analyse the bioactive compounds.

Cell growth inhibition assay

Human breast cancer cell lines (MCF-7) and human cervical cancer cell lines (HeLa) were procured from National Centre for Cell Science, Pune, India. The cells were maintained in Dulbecco's Modified Eagle Medium (Sigma-Aldrich, St. Louis, MO) containing 10% (v/v) foetal bovine serum (Gibco, Thermo Fisher Scientific), 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 2.5 μg ml^{-1} amphotericin, in a humidified incubator with 5% CO_2 at 37 °C. A well-known 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-

bromide (MTT) assay was carried out to assess the effect of fungal extract on the growth of the cancer cell lines. In brief, approximately 2×10^4 cells per well were seeded in 96 well culture plate and incubated overnight. After 16 h, varying concentrations (250–1500 μg ml^{-1}) of fungal extracts were added to the wells. After 72 h of incubation, the MTT assay was carried out as described by Denizot and Lang (1986). Paclitaxel was used as the positive control at the concentration of 20 μg ml^{-1}. The growth inhibition rate was calculated using the formula (OD is optical density):

$$\text{Inhibition rate} = (\text{Mean OD of control well} - \text{Mean OD of treated cell}/\text{Mean OD of untreated cell}) \times 100$$

The IC_{50} in this assay was defined as the concentration of compounds that resulted in 50% inhibition of growth rate.

Antioxidant assay

The antioxidant potential of the fungal extract was studied by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. Fungal extract (50-μl samples) of different concentrations were mixed with 150 μl of DPPH (100 μM) in methanol in the wells of a 96-well microtiter plate. Ascorbic acid (100 μg ml^{-1}; 50 μl) was used as the positive control. The plate was incubated in the dark (45 min), and absorbance of the reaction mixture was measured at 517 nm using an enzyme-linked immunosorbent assay reader (Infinite microplate reader; Tecan Austria GmbH, Grödig, Austria). The percentage inhibition of DPPH radical by the fungal extract was expressed as the inhibition concentration (IC_{50}) and was calculated using the formula:

$$\text{DPPH scavenging activity } (\%) = \left(A_{\text{Control}} - A_{\text{Sample}}/A_{\text{Control}}\right) \times 100$$

Identification of bioactive compounds by UHPLC-QTOF analysis

For the UHPLC-QTOF-MS/MS analysis, the residue was first dissolved in methanol. Chromatographic separation was carried out using the Agilent 1200 UHPLC system equipped with C18 column (Agilent Technologies, Santa Clara, CA). Samples (5 μl) were injected into the column, and elution was carried out in a gradient mode with acidified water (0.2% v/v formic acid) to 90% methanol over a period of 40 min at a flow rate of 0.3 ml min^{-1}. High-resolution mass spectroscopic detection was performed using Bruker Impact QTOF mass spectrometer (Bruker Corp., Billerica, MA, operating in Top5 data-dependent mode from 100–1500 m/z with electron spray ionization, and both positive and negative ions were detected. Smart Formula 3D™ and Fragment Explorer part of Data Analysis 4.2 (Bruker Corp.) were used to generate compound formulae.

Statistical analysis

All the experiments were performed in triplicate. The results were expressed as mean ± standard deviation. The data were analysed by analysis of variance, and the means were compared by Tukey's test at $p < 0.05$.

Results

Isolation and identification of fungal strain T1

The PCR product of ITS-rDNA amplified with ITS1 and ITS4 was 588 bp. BLAST analysis revealed 98% similarity (query coverage of 100%) with *Diaporthe* sp. Bayesian analysis of ITS sequences of different species of *Diaporthe* yielded a consensus tree (Fig. 1), and the present isolate was clustered with *Diaporthe* sp. (KC357558 and KC357559).

Cytotoxic effect in cancer cell lines

The fungal extract showed a cytotoxic effect against both the human breast cancer cell line (MCF-7) and the human cervical cancer cell line (HeLa). The cytotoxic effect become significantly pronounced at higher concentrations of the fungal extract (Fig. 2). The IC_{50} value of the extract was 1058 ± 44 and 1257 ± 80 μg ml^{-1} for the MCF-7 and HeLa cell lines, respectively. Paclitaxel (20 μg ml^{-1}), an anticancer drug, was used as a positive control. It inhibited the growth of the MCF-7 and HeLa cell lines by 79 ± 6 and 97 ± 2%, respectively.

Antioxidant activity

Free radical scavenging activity was performed to determine the antioxidant activity of *Diaporthe* sp. The scavenging activity significantly increased with increasing concentration of fungal extract (Fig. 3). The IC_{50} value (concentration of sample required to scavenge 50% of free radicals) of the crude extract was 250 ± 3.5 μg ml^{-1}. Ascorbic acid, the positive control, showed 84 ± 3.5% antioxidant activity.

Identification of compounds by UHPLQTOF analysis

The UHPLC-QTOF- MS/MS analysis of the crude extract of *Diaporthe* sp. (T1) showed a signal (*m/z*) corresponding to three trichalasins. Figure 4 shows the mass data of these three compounds that eluted from the column at different times.

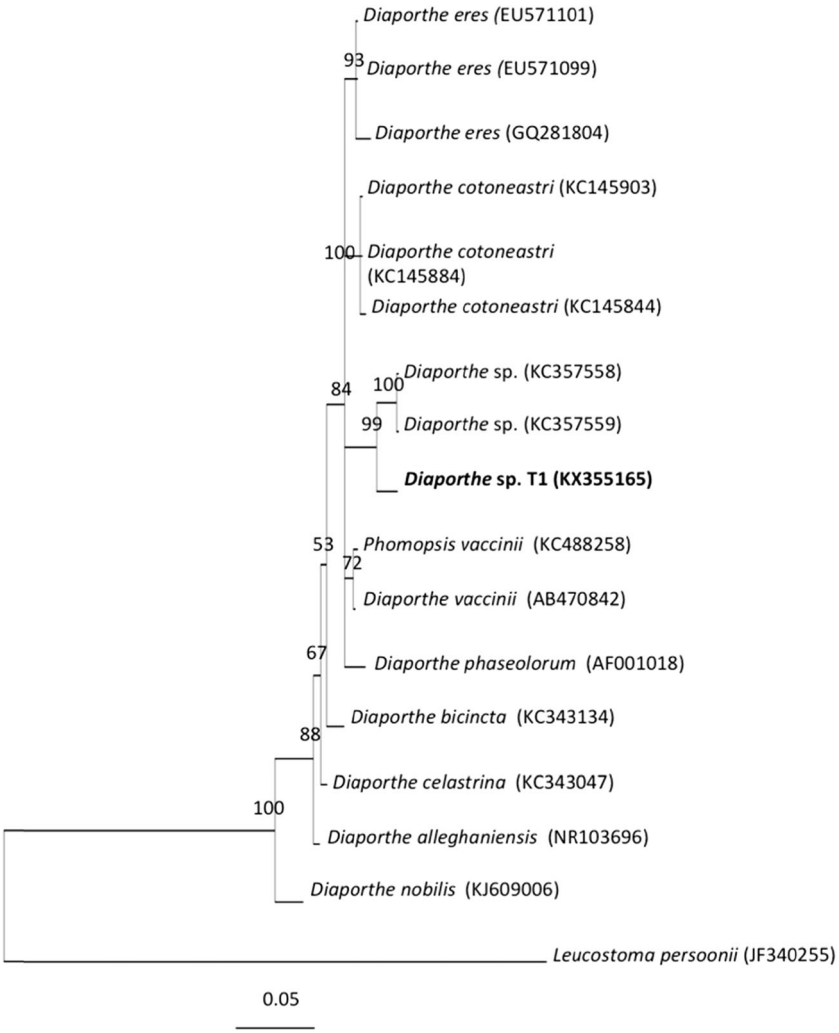

Fig. 1 Bayesian tree showing the relationships between the internal transcribed spacer (ITS) sequence of *Diaporthe* sp. (shown in *bold*) and those of related species retrieved from GenBank. *Numbers at nodes* Posterior probability percentages (>50%) of the Bayesian analysis

Peaks at *m/z* ratios of 402.1753, 420.1391 and 472.1511 were observed and identified as Trichalasin H, Trichalasin F) and Trichalasin E, respectively, based on the reported values of these compounds (Electronic Supplementary Material Table).

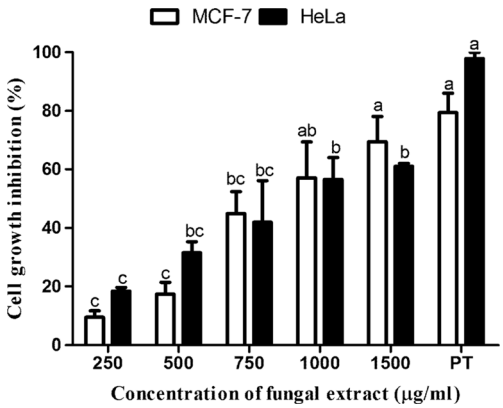

Fig. 2 Cytotoxic effect of *Diaporthe sp*. extract against human breast cancer (*MCF-7*) and human cervical (*HeLa*) cancer cell lines. *Bars* Mean values, *error bars* standard deviation (SD). *Bars with the same lowercase letter* within the same cell type are not significantly different at $p < 0.05$. *PT* Paclitaxel (20 μg ml^{-1}) used as the positive control

Discussion

The anamorphic form (the asexual reproductive morphological stage) of *Diaporthe* is the genus *Phomopsis*. Members of *Diaporthe*/*Phomopsis* are either plant pathogens or endophytic species (van Niekerk et al. 2005; Dos

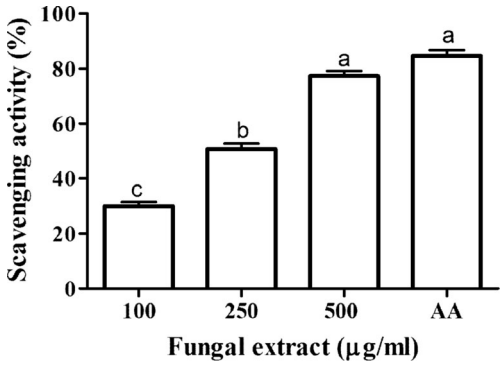

Fig. 3 Antioxidant effect of *Diaporthe* sp. extracts based on free radical scavenging activity. *Bars* Mean values, *error bars* standard deviation (SD). *Bars with the same lowercase letter* are not significantly different at $p < 0.05$. *AA* Ascorbic acid (100 μg ml^{-1}) used as the positive control

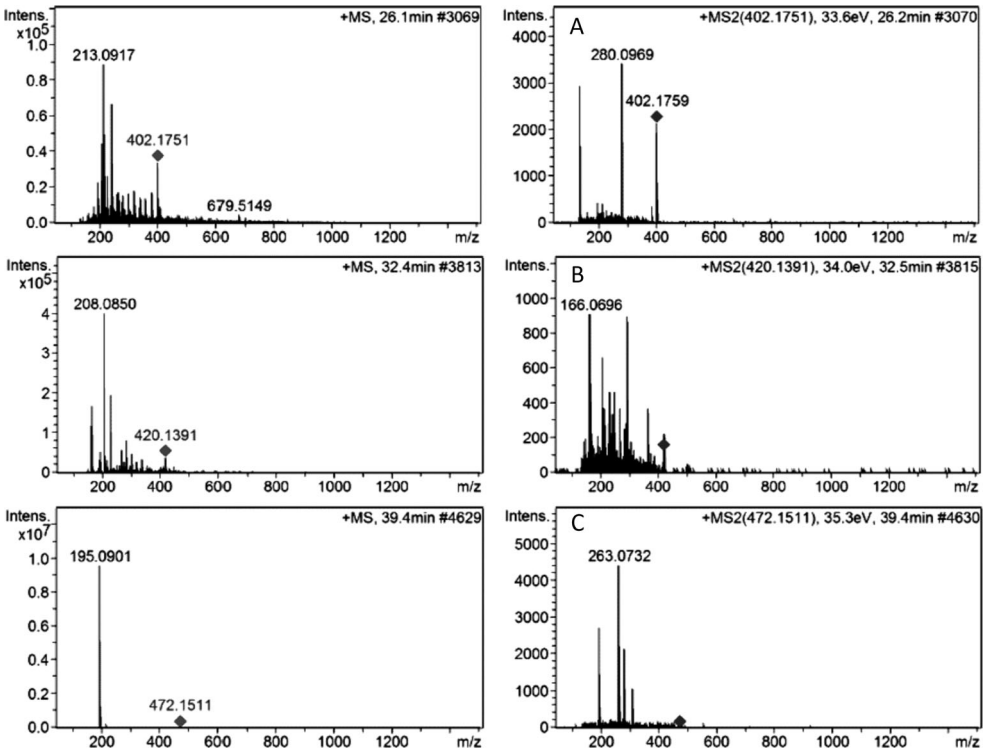

Fig. 4 The tandem mass spectrometry profile of selected ions in the extracts of *Diaporthe* sp. T1 corresponding to three tricalasins: **a** Trichalasin H (*m/z* 402.1753), *b* Trichalasin F (*m/z* 420.1391), **c** trichalasin E (*m/z* 472.1511)

Santos et al. 2016) and are associated with dicotyledonous and monocotyledonous plants of tropical and temperate regions (Eriksson and Vue 1998; Guo et al. 2000). The *Diaporthe*/*Phomopsis* complex occurs as endophytes with medicinal plants such as *Taxus chinensis* (Liu et al. 2009), *Taxus globosa* (Soca-Chafre et al. 2011) and some medicinal shrubs of India (Naik et al. 2008). Here, we report for the first time an endophytic association of a *Diaporthe* sp. with *Taxus baccata* subsp. *wallichiana*.

Medicinal plants are a repository of endophytic fungi that are able to produce bioactive compounds of biotechnological and medicinal interest (Vieira et al. 2012). We recovered a *Diaporthe* species from *T. baccata* as an endophyte that produced metabolites displaying cytotoxic activity against MCF-7 and HeLa tumour cells. Agusta et al. (2006) isolated (−)-epicytoskyrin from a *Diaporthe* species isolated from the tea plant and found that this compound exhibited moderate cytotoxic activity against KB cells, a subline of HeLa cells, with an IC_{50} value of 0.5 μg ml^{-1}. Various researchers have reported the cytotoxic activity of the extracts of *Diaporthe* species against different cell lines (Lin et al. 2005; Carvalho et al. 2012; Casella et al. 2013). In our study, the culture filtrate of *Diaporthe* sp. showed antioxidant activity when assayed using the DPPH method. The DPPH assay has been extensively used as a reliable method to measure antioxidant activity of pure compounds (Koleva et al. 2002). Our results suggest that metabolites of *Diaporthe* sp. could serve as potential agents in scavenging free radicals. Antioxidant activity of the *Diaporthe*/*Phomopsis* complex has been reported by Nath et al. (2012) and Ascêncio et al. (2014).

Cytochalasins are a known class of mould metabolites that exhibit a wide range of distinctive biological activities. They are implicated as phytotoxins or virulence factors and exhibit antimicrobial and cytotoxic activities (Wagenaar et al. 2000). Cytochalasins are produced by a variety of fungal genera, including *Phomopsis*, *Aspergillus*, *Penicillium* and *Chaetomium* (Yan et al. 2016). Structurally, cytochalasins are comprised of a highly substituted isoindolone ring with a benzyl group at the C-3 position and fused to an 11- to 14-member macrocyclic ring. Wagenaar et al. (2000) reported the isolation of four cytotoxic cytochalasins from a culture of the endophytic fungus *Rhinocladiella* sp., and Yan et al. (2016) identified four cytochalasins (phomopchalasin A, B, C and J) from the endophytic fungus *Phomopsis* sp. isolated from the stem of *Isodon eriocalyx* var. *laxiflora*. Pornpakakul et al. (2007) isolated diaporthichalasin, a cytochrome P450 3A4 inhibitor, from an endophyte *Diaporthe* species isolated from *Croton sublyratus*. Chen et al. (2014) reported the production of three new cytochalasins (trichalasin E, F and H) along with four analogues from the endophytic fungus *Trichoderma gamsii* residing in the Chinese medicinal plant *Panax notoginseng*. In the present study, we identified three cytochalasins (trichalasin E, F and H) from *Diaporthe* sp. and confirmed their identity based on their *m/z* values. The biological activities exhibited by this fungus may be correlated with the production of these bioactive compounds (trichalasins). This is the first report of the

occurrence of trichalasins (E, F and H) from an endophyte *Diaporthe* sp. isolated from *T. baccata* subsp. *wallichiana*.

Our results show that the culture extracts of *Diaporthe* sp. exhibited strong cell growth inhibition and antioxidant properties, indicating the ability of this fungus to produce bioactive compounds that may be useful as sources of novel drugs. Our work also confirms the production of trichalasins, which provides an insight into understanding some basis of therapeutic properties of fungal endophyte *Diaporthe* sp. Further studies on the isolation and characterization of these trichalasins and their in vivo assays will be required to assess the biological function of these compounds.

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