Metabolic profiling and biological activity of *Pseudocercospora hakeae* on *Curculigo orchioides* Gaertn.

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Plants have an endophytic association with a diverse group of fungi that help them accumulate compounds of therapeutic importance. In this study, we highlight the significant role of a genetically identified endophyte, *Pseudocercospora hakeae* from *Curculigo orchioides* in providing potent medicinal property to the host plant assessed through its metabolic profiling, various antioxidant activities as well as enzyme production. HPTLC profile of ethyl acetate extract, and quantification of total phenolic and total flavonoid contents proved significant production of secondary metabolites.

Keywords: Antioxidants, *Curculigo orchioides*, enzyme production, metabolic profiling, *Pseudocercospora hakeae*.

PLANTS are being exploited for certain therapeutically important compounds which are actively involved in their protection against biotic and abiotic stresses. Some plants have endophytic associations, where the endophyte produces the same bioactive compounds as reported from the host plants. A classic example is the endophytic fungus *Taxomyces andreanae* from *Taxus brevifolia* producing taxol[1].

Curculigo orchioides Gaertn. (family Amaryllidaceae) is an endangered herb native to India, popularly known as 'kali musli'. It is a potent immunomodulator and aphrodisiac in the Ayurvedic medicinal system[2] with anticancer, anti-diabetic, anti-neurodegenerative and hepatoprotective activities[3]. Most of the medicinal properties of this host plant are contributed by the characteristic phenolic glycoside, curculigoside, along with other secondary metabolites. The wild populations of the plant have been considerably depleted due to various reasons such as commercial exploitation, destruction of habitats and poor seed set[4,5]. Hence endophytes of *C. orchioides* may serve as an alternative to the plant and contribute towards conserving the valuable resources. Endophytic diversity of *C. orchioides* was explored in a few works only[6]. We selected the endophyte isolated from the leaves of *C. orchioides*, which showed maximum antibacterial potential among the isolates in our unpublished data, for its identification and evaluation of other biological activities.

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Materials and method

Identification of the potent endophyte

The potent endophyte was identified based on the colony and hyphal morphology of the fungal culture and further confirmed by molecular identification. For genomic-level identification, genomic DNA of the endophytic fungus was isolated using Chromous Biotech gDNA Mini spin kit. The target regions of the rDNA ITS1, ITS2 regions and the 5.8S gene were amplified asymmetrically using universal ITS primers, ITS1 (5′-TCC GTA GGT GAA CCT GCGG-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′), as described by White *et al.*[7] in a ProFlex™ (applied biosystems) PCR system under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55.5°C for 30 sec, extension at 72°C for 2 min and a final extension at 72°C for 10 min. The amplification was confirmed by agarose gel electrophoresis and the amplicon was sequenced using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems) employing the same primers for amplification. The ITS sequence thus obtained was subjected to BLAST analysis to identify its similarity to the reported sequences. The sequence of putative fungus and other related sequences were multiple-aligned using BioEdit version 7.2.6.1. The ITS sequence thus obtained was submitted to GenBank. Phylogeny was analysed by N–J method in MEGA X.

Mass cultivation of endophytic fungi

The genetically identified endophyte was slant-cultured and maintained in our laboratory. Agar blocks of actively growing pure culture (3 mm in diameter) were placed on

potato dextrose broth in 500 ml Erlenmeyer flasks containing 200 ml of the medium for cultivation en-masse[8]. The flasks were incubated at room temperature for three weeks with periodical shaking at 150 rpm. After the incubation period, the mycelial mats were removed from the cultures by filtering through sterile cheesecloth. These mats were dried and the biomass of endophytes was measured.

Extraction of metabolites from endophytic fungi

The extraction was carried out according to the protocol mentioned by Raviraja *et al*.[9]. After mass cultivation of endophytic fungus, the fungal metabolites from endophytic mycelial mats were extracted using ethyl acetate (EA). An equal volume of the filtrate and solvent was taken in a separating funnel and shaken vigorously for 10 min. The solution was then allowed to stand; the cell mass got separated and the solvent thus obtained was collected. EA was evaporated to yield the crude extract.

Metabolic profiling

Various qualitative chemical tests were performed with EA extract of *P. hakeae* to determine phytochemical constituents such as alkaloids, flavonoids, terpenoids, coumarins, cardiac glycosides, tannins and phlobatannin using standard methods[10,11].

Total phenolic content and total flavonoid content in the crude extract of *P. hakeae* were determined according to the standard protocol[12] and the results were expressed as gallic acid and quercetin equivalents respectively.

For HPTLC analysis of alkaloids the sample solutions were spotted in the form of bands having a width of 8.0 mm using a Camag microlitre syringe on per silica gel aluminium-coated plate 60 F 254 (100 mm × 100 mm, Merck) using a Camag Linomat V (Switzerland) with a constant application rate of 150 nl/s. The slit dimension was kept at 5.0 mm × 0.2 mm and 20 mm/s scanning speed was used. Linear ascending development was carried out in 20 cm × 10 cm twin-trough glass chamber (Camag, Switzerland) saturated with Whatman No. 1 filter paper in the mobile phase EA (10) : methanol (1.3) : water (1) for the optimized chamber saturation time of 20 min at room temperature. The solvent front position was noted as 70 mm. After the development, TLC plates were dried for 5 min at room temperature for complete removal of the mobile phase. The plates were kept in a photodocumentation chamber (Visualizer: S/N: 241537) and the images were captured at visible light, UV 366 nm and 254 nm to identify the peak numbers with their height and area, peak display and peak densitogram. Densitometric scanning was performed using a Camag TLC scanner IV (S/N: 241072) in the reflectance absorbance mode at 254 nm and utilizing vision CATS software with the help of deuterium and tungsten lamp. The TLC plates were dipped in Dragendorff reagent followed by drying in an oven at 100°C for 3 min. The R_f value was calculated: R_f = distance travelled by the substance/distance travelled by the solvent.

Antioxidant activity was evaluated by three different methods – phosphomolybdenum-based assay for total antioxidant activity[13], reducing power assay by the modified method of Oyaizu[14] and diphenyl picryl hydrazyl (DPPH) radical scavenging activity according to Babu and Rao[15]. Results were expressed as milligrams of ascorbic acid equivalents per gram of extract.

Qualitative determination of protease, lipase and amylase was done according to the reported protocols[16,17]. The functional role of exoenzymes was indicated by measuring (mm) the halo zones surrounding the fungal colony.

All the experiments were carried out in triplicate and mean ± SD was determined statistically using SPSS version 22. Data were analysed by ANOVA, considering $P < 0.05$ as significant. Graphs were plotted with Excel 2013.

Results

Morphological and molecular identification of the endophytic fungus

The fungal mycelium was brownish with white entire margins and mycelial mat appressed to the medium. The homology search of 500 bp amplicon sequence of endophytic fungus exhibited 97.58% similarity with the sequence of a previously identified fungus, *Pseudocercospora hakeae* at the nucleotide level in BLAST at the NCBI site (Figure 1). The sequence obtained was deposited in GenBank with accession number MW024066. *P. hakeae* formed a separate cluster with the other reported endophytic *Pseudocercospora* species in the phylogenetic tree (Supplementary Figure 1).

Metabolic profiling

The identified endophyte, *P. hakeae* was maintained by regular subculturing and then it was mass cultured for secondary metabolite extraction (Figure 2). Upon EA extraction we got 193.6 mg/litre extract.

Phytochemical analyses of EA of *P. hakeae* unveiled the presence and absence of secondary metabolites such as alkaloids, flavonoids, terpenoids, saponin, phenol, coumarin, cardiac glycoside, tannins and phlobatannin (Table 1).

The results from HPTLC for alkaloids can be distinguished at UV 254 nm before derivatization (Figure 3). The EA extract showed two dark spots at 254 nm and two blue spots at 366 nm in the chromatogram. Their corresponding R_f values were 0.073, 0.289, 0.679 and 0.924

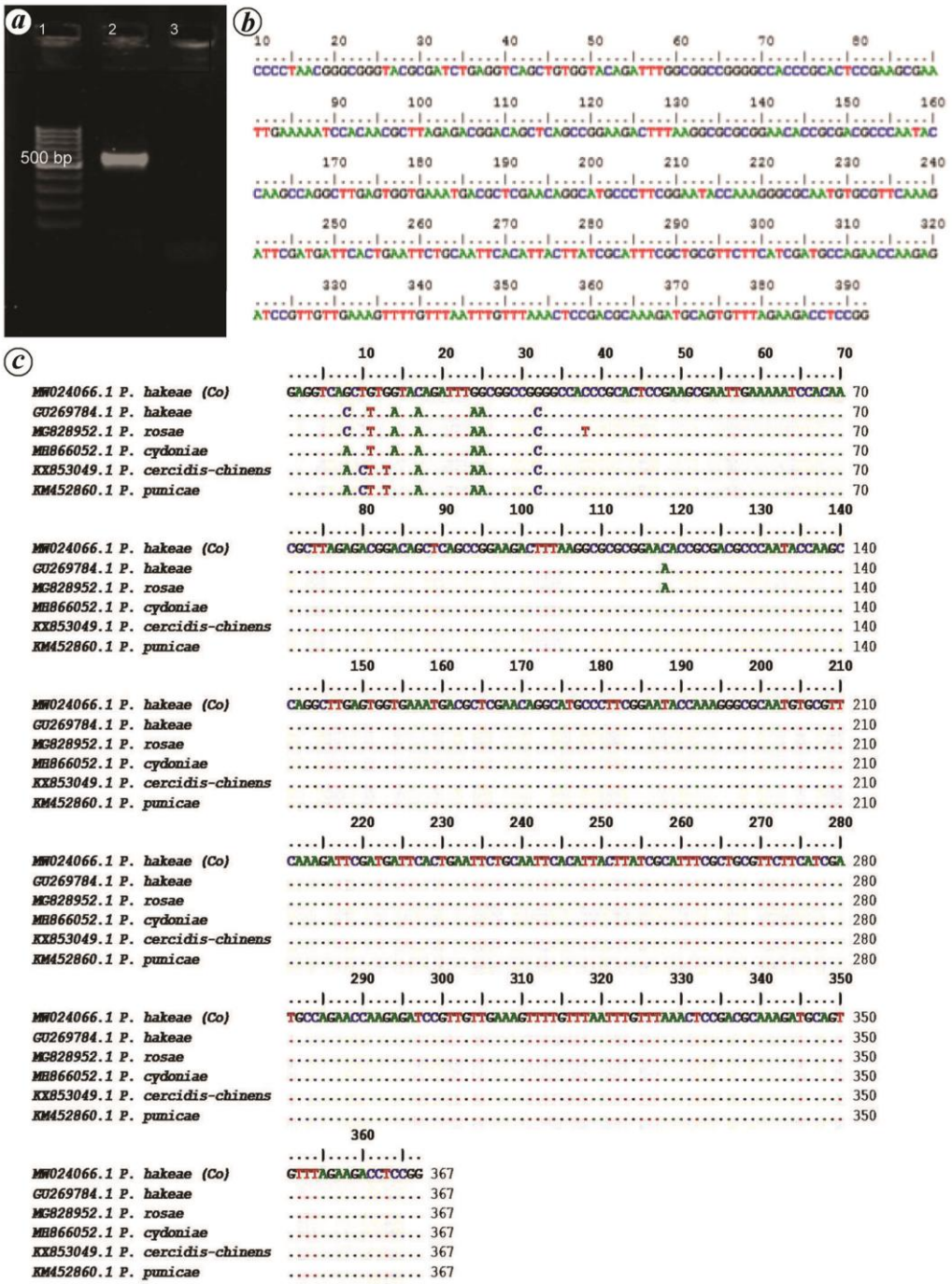

Figure 1. Molecular identification of *Pseudocercospora hakeae*. ***a***, PCR amplification of the ITS region from *P. hakeae* on 1% (w/v) agarose gel. Lane 1, 100 bp DNA marker; Lane 2, PCR product; Lane 3, PCR control. ***b***, Nucleotide sequence of the ITS region obtained from *P. hakeae*. ***c***, Multiple sequence alignment of nucleotide sequences of various species of *Pseudocercospora* with isolated *P. hakeae* (co-*Curculigo orchioides*). Accession numbers are indicated on the right-side of selected sequences. Identical sequences are represented as single dots.

respectively. As shown in Table 2, the peak with R_f value of 0.679 had maximum percentage area of 41.16 in the densitogram followed by the peak with 36.08% area having an R_f value of 0.073. The densitogram area of 20.30% was covered by the peak with R_f value of 0.924 and the area least covered was 2.46% by the peak with R_f value of 0.289. Thus, the preferred profile may have been due to the mobile phase of EA : methanol : water (10 : 1.3 : 1).

The antimicrobial activity observed in our previous study along with other reported studies can be associated with the presence of phenolic and polyphenolic compounds[18]. Quantitative estimation of phenols and flavonoids was therefore done to substantiate the antibacterial activity

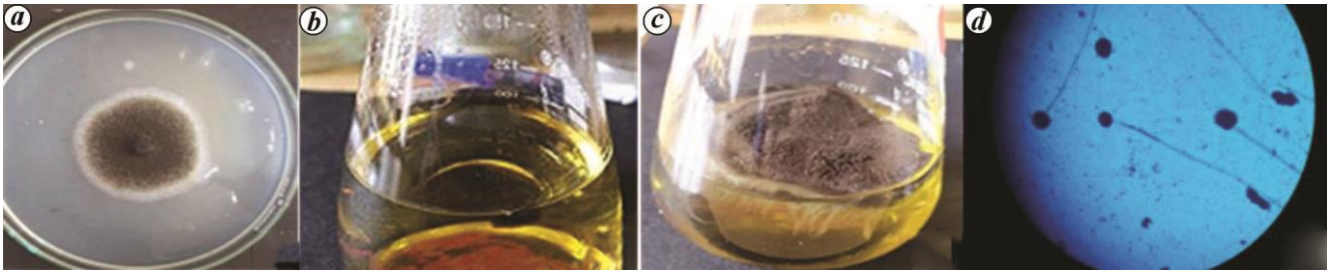

Figure 2. *P. hakeae* isolated from the leaves of *C. orchioides*. ***a***, Subcultured fungi grown in potato dextrose agar. ***b***, Inoculated fungi in potato dextrose broth. ***c***, Mass culture of endophytic fungi after 14 days. ***d***, Microscopic view of fungal hyphae.

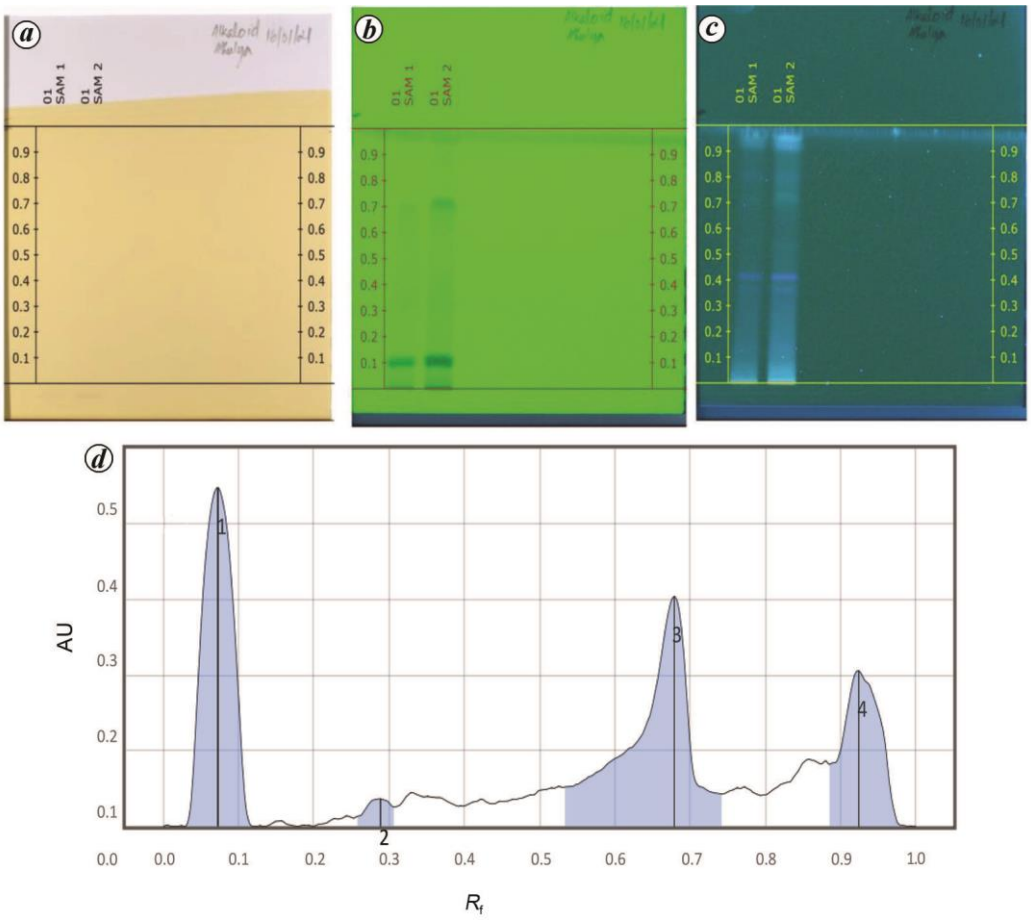

Figure 3. HPTLC fingerprinting profile of ethyl acetate (EA) extract of *P. hakeae* for alkaloids. Chromatograms at different wavelengths: (***a***) visible range, (***b***) R 254 nm and (***c***) R 366 nm (SAM 1 – sample of volume 5.0 µl, SAM 2 – volume of 10.0 µl), (***d***) Densitogram showing peaks of different compounds. AU, Unit area of peak; R_f, retardation factor.

(Table 3). Total phenol content (44.5 ± 0.010 mg/g of gallic acid equivalent) and total flavonoid content (24.9 ± 0.010 mg/g of quercetin equivalent) obtained were sufficient to induce biological activity and are relatable from the literature.

Antioxidant activity of the endophytic extract

P. hakeae studied with respect to its antioxidant property by three different methods – phosphomolybdenum-based total antioxidant activity measurement, reducing assay and radical scavenging activity using DPPH. We calibrated 0.247 ± 0.113 mg/g of the ascorbic acid equivalent of total antioxidant activity from the standard graph by phospho-molybdenum-based assay (Figure 4). The change of ferric to ferrous form due to hydrogen ions provided by the antioxidants in the endophytic extract was evaluated. We observed high absorbance of 0.336 at 500 µg/ml concentration and calibrated the reductive ability as 71.05 ± 1.25 mg/g of ascorbic acid equivalent, and obtained

Table 1. Phytochemical analysis of ethyl acetate extract of *Pseudocercospora hakeae*

Phytochemical test	Method	Observations	*P. hakeae* crude
Alkaloids	Add 2–3 drops of Dragendorff's reagent to 1 ml of crude extract	Turbidity or precipitation	+
Flavanoids	Add 2–3 drops of NaOH to 1 ml of endophyte extract	Appearance of yellow colour	+
Phenols	Add a few drops of 1% $FeCl_3$ to 1 ml of crude extract	Appearance of red-brown colour	+
Tannins	Add 2–3 drops of $FeCl_3$ to 1 ml of crude extract	Appearance of blackish-blue or blackish-green colour	+
Terpenoids	Salkowski test: mix 1 ml of chloroform with 2.5 ml of crude extract and carefully add 1.5 ml of H_2SO_4	Appearance of reddish-brown coloured interface	+
Saponins	Add 2–3 drops of olive oil to 5 ml of endophyte extract and shake vigorously	Froth formation	+
Phlobatannin	Add 2 ml of crude extract into dilute HCl	Red precipitate	–
Cardiac glycoside	Alcoholic solution of the extract mix with a few drops of NaOH and 2% 3,5-dinitrobenzoic acid	Appearance of pink colour	–
Coumarins	Crude extract mix with 1 N NaOH solution	Blue-green fluorescence	–

+, Indicates the presence of compounds; –, indicates the absence of compounds.

Table 2. HPTLC fingerprinting profile for alkaloids of ethyl acetate extract of *P. hakeae*

	Start		Maximum			End					
Peak no.	R_f	*H*	R_f	*H*	Percentage	R_f	*H*	Area *A*	Percentage	Manual peak	Substance
1	0.029	0.0000	0.073	0.4480	45.07	0.121	0.0000	0.02133	36.08	No	Unknown
2	0.253	0.0105	0.289	0.0363	3.66	0.306	0.0255	0.00145	2.46	No	Unknown
3	0.531	0.0511	0.679	0.3041	30.59	0.742	0.0424	0.02433	41.16	No	Unknown
4	0.885	0.0819	0.924	0.2057	20.69	0.990	0.0002	0.01200	20.30	No	Unknown

R_f, Retardation factor; *H*, Height equivalent of a theoretical plate.

Table 3. Quantitative analysis of ethyl acetate extract of *P. hakeae*

Secondary metabolites	Content
Total phenol content	44.5 ± 0.010 mg/g GAE
Total flavonoid content	24.9 ± 0.010 mg/g QE

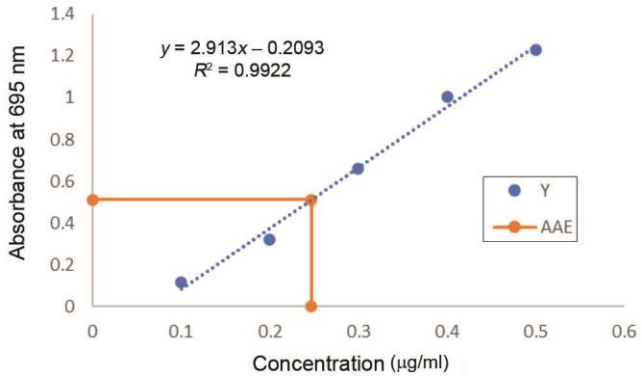

Figure 4. Standard curve for total antioxidant activity of crude extract of *P. hakeae* by phosphomolybdenum assay. Each value is expressed as mean ± standard deviation (n = 3).

97.87% of inhibition at 100 µg/ml concentration (Figure 5). The capability to reduce DPPH radicals induced by antioxidants was determined at 517 nm. *P. hakeae* showed maximum scavenging of 85% at 250 µg/ml concentration (Figure 6) and the obtained IC_{50} value was 103.632 µg/ml.

Enzymatic activity

Various industries such as textiles, cosmetics, beverages, foods, etc. depend on specific enzymes to improve product quality. The diversity of microbial enzymes increases with the discovery of enzymes and their applications[19]. Enzyme production by endophytic fungi may vary, as it is related to host–fungus specificity. In the present study, we observed the production of proteases and amylases and also the absence of lipolytic enzymes (Figure 7).

Discussion

Many plants have a symbiotic association with endophytic fungi that helps them to accumulate secondary metabolites which in turn provide them shelter. In our previous work, we isolated a total of seven endophytic fungi, five from the leaves and two from the tuber of *C. orchioides* (unpublished data). Among them, the endophyte showing potent antibacterial activity against both Gram-positive and Gram-negative bacteria was identified as *P. hakeae* by similarity search of the ITS sequence region (GenBank accession number MW024066). According to Ling

et al.[20], *Pseudocercospora* species from *Fagopyrum tataricum* exhibited maximum antibacterial activity and they recommended the fractionation, purification and identification of the antimicrobial metabolites from this endophyte. Prihantini and Tachibana[21] reported the production of antioxidant compounds, terreic acid and 6-methyl salicylic acid by the endophyte *Pseudocercospora* sp. ESL 02 in *Elaeocarpus sylvestris*. In the phylogenetic lineages in *Pseudocercospora*, Crous *et al.*[22] referenced *P. hakeae* as an endophyte from the leaves of *Hakea salicifolia* and the leaves of *Grevillia* species. In this study, we report *P. hakeae* from *C. orchioides.*

We obtained a considerable amount of extract (0.484 g) from 2.5 litre culture filtrate. The biological activities of endophytes are contributed by the secondary metabolites produced by them. Preliminary screening in *P. hakeae* showed the presence of the principal secondary metabolites produced by its host plant. Phenolics act as active antioxidants and the most important stabilization factors of the oxidative processes and play a significant role in defence against pathogens and various environmental stresses[23,24]. Tannins exhibit antibacterial activity[25] that inhibits protein synthesis[26,27] by binding with proline-rich proteins. Aromatic compounds, terpenoids and glycosidic saponins also possess antibacterial ability. In response to microbial infections, plants that produce flavonoids are found to have *in vitro* antimicrobial activity[28]. The presence of these secondary metabolites and the *in vitro* biological activities exhibited by the endophyte supports the view that the secondary metabolites contribute towards its biological activity.

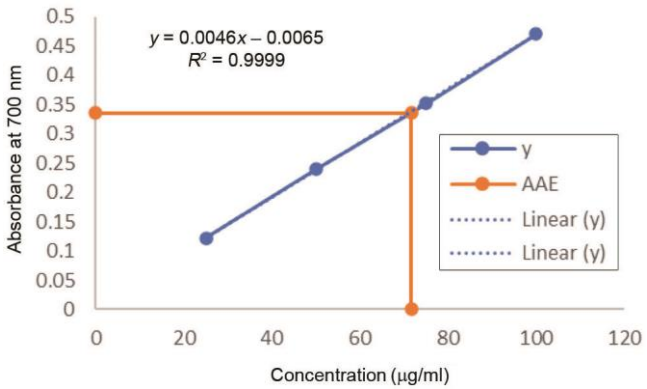

Figure 5. Standard curve for reducing assay of EA extract of *P. hakeae*. Each value is expressed as mean ± standard deviation (n = 3).

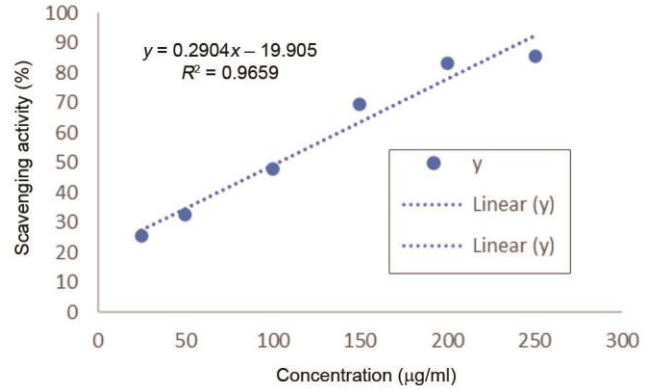

Figure 6. Graphical representation of scavenging ability of EA extract of *P. hakeae* against diphenyl picryl hydrazyl (DPPH) radicals. Each value is expressed as mean ± standard deviation (n = 3), P < 0.05.

Salome-Abarca *et al.*[29] used the metabolic profile of fungal endophytes from *Alstonia scholaris* and *Euphorbia myrsinites* by HPTLC to study biotransformation of plant metabolites by the endophytes. In the present study, qualitative analysis through HPTLC fingerprinting confirmed the presence of alkaloids in the EA extract of *P. hakeae*. Alkaloids are the nitrogen sources found in plants that are considered to have several medicinal properties like anti-inflammatory, analgesic and adaptogenic. These compounds have a significant role in developing resistance against diseases and stress[30]. Four types of alkaloids with different R_f values determined in the EA extract of *P. hakeae* show that this is an alkaloid-rich endophyte. The area of all the compounds separated and shown in the densitogram (Figure 4 and Table 2), suggests ample production of secondary metabolites of nitrogen origin by the endophyte *P. hakeae.* HPTLC could be used as an effectual approach to track the endophyte–host plant interactions.

Since the antioxidant properties of *C. orchioides* were reported to be partly attributed to the phenolics and phenolic glycosides present, we studied the total phenolic content and total flavonoid content in the isolated *P. hakeae.* Both were modest in the present study and this may add a considerable amount of phenol and flavonoid contents in *C. orchioides*, which showed total phenolic and total flavonoid contents of 196.34 ± 1.45 mg gallic acid equivalent (GAE)/g and 78.49 ± 1.78 mg QE/g respectively[31]. High amount of phenolics (112 ± 4.12 mg/g GAE) was found in EA extract of *Penicillium citrinum* CGJC2 isolated from *Tragia involucrate* L.[32]. Similarly, 18.33 ± 0.68 mg GAE/g of total phenol content and 6.44 ± 1.24 mg quercetin equivalent per gram of total flavanoid content were quantified in *Phyllosticta* sp. isolated from *Gauzuma tomentosa*[33].

Three different assays were used for evaluating antioxidant potential, of which DPPH is the most widely used and most accurate screening method[34]. In reducing assay, free radicals are reduced by hydrogen ions that are donated by the antioxidants[35]. Antioxidant property of many endophytic fungi from different medicinal plants has been evaluated. EA extract of *Aspergillus terreus* isolated from *Ocimum sanctum* exhibited 34.83% antioxidant activity[36]. Prihantini *et al.*[21] reported the highest antioxidant activity (IC_{50} = (30.54 ± 0.88) mg/ml) in *Pseudocercospora* sp. ESL2 from *Elaeocarpus sylvestris* and isolated antioxidant compounds from it. EA extract of *Aspergillus flavus* in *Hibiscus subdariffa* showed 2.71 ± 0.12 mg/ml concentration of total antioxidant activity[37]. Choudhary *et al.*[38]

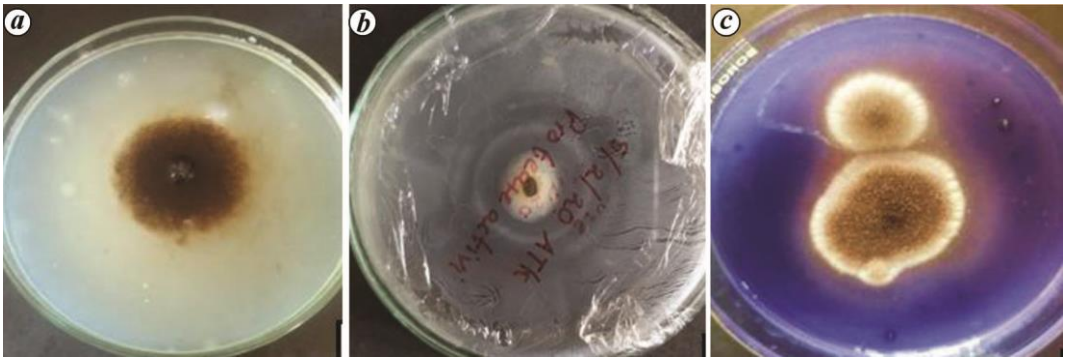

Figure 7. Representative images of enzyme production by *P. hakeae.* ***a***, Lipase activity; ***b***, protease activity; ***c***, amylase activity.

reported 0.62 ± 0.05 and 0.39 ± 0.04 TAC by *Aspergillus neoflavipes* and *Penicillium singorense* isolated from *Calotropis procera* respectively.

Our results with all three antioxidant assays indicate that the endophytic fungal isolate obtained from the traditionally used ethnomedicinal plant, *C. orchioides* possesses different groups of antioxidants that are comparable or even better in terms of their potency with standard antioxidants and may be useful as microbial cell factories for the production of antioxidants in future.

As part of host adaptation, fungal endophytes produce enzymes such as amylases, lipases and proteases that help them invade the host plant and obtain nutrients for their growth. An endophytic fungus *Fusarium* sp. on Chrysanthemum[39] produced a highly active fibrinolytic enzyme[39]. More extensive studies are needed to optimize enzyme synthesis and evaluate the potential of endophytic fungi as a source of industrial biocatalysts[40]. We add one more member *P. hakeae* to the list of endophytes as producers of enzymes with amylolytic and proteolytic activities.

Conclusion

This study gives an insight into the secondary metabolite production, antioxidant capacity and enzymatic potential of the endophyte *P. hakeae* recorded from the medicinally important plant *C. orchioides*. It is a fast-growing fungus rich in secondary metabolite production. Significant production of phenols, flavonoids and alkaloids was noticed, and confirmed by both quantitative and HPTLC analysis. It was found to possess significant antibacterial, antioxidant and enzymatic activities that might be exploited for therapeutic uses. Thus, this study paves the way for further research on secondary metabolite production by endophytes from *C. orchioides* to reduce reliance on its host plant for therapeutic uses to ensure sustainable development.

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ACKNOWLEDGEMENT. We thank the University Grants Commission, New Delhi for providing grants under the UGC-JRF programme.

Received 29 September 2021; revised accepted 20 December 2021

doi: 10.18520/cs/v122/i3/290-297