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ORIGINAL ARTICLE

Bioactive sesquiterpene, plasticizer, and phenols from the fungal endophytes of *Polygonum chinense* L.

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Abstract

There is a constant need for novel antibiotic and antioxidant sources due to the ever-increasing resilience of pathogens and the occurrence of chronic diseases. The natural sources of these agents have advantages due to lower production cost, structural variation, and uses of active compounds for pharmaceutical uses. The microbes living in planta termed "endophytes" are alternative sources of host bioactive compounds. In this study, ten endophytic fungi were isolated from *Polygonum chinense* L. and identified by sequencing of the internal transcribed spacer regions. The fungal strains were fermented and the ethyl acetate extracts were evaluated for antimicrobial and antioxidant capacities. Almost 80% of the endophytes showed antibacterial potency against one or more pathogenic bacteria. Among all strains, *Penicillium canescens* showed broad-spectrum antimicrobial activity against gram-positive and gram-negative pathogens as well as significant antioxidative and DNA protective capacities. The strain *Fusarium chlamydosporum* displayed significant anti-radical (126.8 ± 6.7 μg/ml) and ferric reducing (84.7 ± 2.1 mg AA/g dry extract) capacities. The bio-autography, chromatography, and mass spectroscopy analyses of *P. canescens* extract revealed the presence of sesquiterpene (germacrene), plasticizer (phthalic acid ester) along with phenolic acids, flavonoid (quercetin), and short chain hydrocarbons. The secondary metabolites of *F. chlamydosporum* were identified with phenolic acids as bioactive compounds by chromatography and mass spectroscopy. This study indicates *P. chinense* endophytes as potential sources of antimicrobial and antioxidant compounds for novel drug discovery.

Keywords Endophytic fungi · Antimicrobial · Bio-autography · Germacrene · Phthalic ester · Antioxidant phenolics

Introduction

Medicinal plants are investigated since long for unique and potential metabolites. In most developing countries, it is considered as the source of pharmaceutical products (Kaul et al. 2012) and therefore are exploited rigorously. The overexploitation of medicinal plants for newer and potential pharmaceutical compounds poses threat to biodiversity.

Endophytes of medicinal plants have incited considerable interest and attention for the wide diversity of bioactive metabolites (Cai et al. 2004; Strobel et al. 2004; Newman and Cragg 2007). Medicinal plants afford unique microcosm for the endophytes that reside in the internal organs or cell spaces of healthy plants. Diverse fungal endophytes exist within plant tissues and each plant can host one or more endophytes (Yu et al. 2010). The endophytes apparently stay in a mutualistic association with the host plants and can be a novel source of metabolites of pharmaceutical interest (Huang et al. 2008a). Endophytes are capable of producing similar secondary metabolites as their hosts (Alvin et al. 2014).

The search for more novel antibiotics of modern times is highly called for as the microorganisms and humans have developed resistance to the existing antibiotics thus posing greater challenge. The fungal endophyte-derived bioactive compounds provide us with new choices of novel antibiotics which can be effectively used against infectious diseases. Many endophytic fungal strains are reported to produce novel broad-spectrum bioactive compounds belonging to alkaloids, macrolides, terpenoids, flavonoids, glycosides, xanthones, isocoumarins, quinones, phenylpropanoids, aliphatic metabolites, lactones, etc. (Kaul et al. 2012). However, the antimicrobial compounds isolated till date from those fungal endophytes are only a small part

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of total endophytic species (Yu et al. 2010). The endophytic fungi produce a plethora of substances for potential use in modern medicine including antioxidants. The antioxidants are important compounds which are potential therapeutic agents against the oxidative damage caused by free radicals. Novel antioxidants have been isolated from a number of endophytic fungi (Kaul et al. 2012).

India is one of the 17 mega-biodiverse countries in the world as recognized by Conservation International (Mittermeier et al. 1997). The Western Ghats, being a biodiversity hotspot in southern India, is a home for copious medicinal plants used ethnomedicinally. *Polygonum chinense* L., commonly known as Chinese knotweed, belongs to the family Polygonaceae and is common in subtropical and warm temperate regions of Asia (Maharajan et al. 2012). Species of *Polygonum* contain pharmaceutically important bioactive compounds (Ismail et al. 2012). The plant itself is used to treat various diseases viz., diarrhea, dyspepsia, hemorrhoid sprains, tonsillitis, and poisonous snake bites (Chevallier 1996). In local health traditions, the decoction of stem and roots mixed with jaggery is given to women for post-natal care. Owing to the traditional usage of this plant in providing health benefits, this plant was selected for the isolation of fungal endophytes from the Talacauvery subcluster of Western Ghats.

The isolation of endophytic fungi from *P. chinense* and the characterization of bioactive compounds are limited, though the plant is reported to possess antimicrobial compounds (Ezhilan and Neelamegam 2012). Hence, the primary aim of the study was to isolate and characterize the fungal endophytes from the plant parts of *P. chinense* and characterization of secondary metabolites of fungi responsible for the antimicrobial and antioxidant potential by chromatographic, bio-autography, and ESI-MS/MS techniques. To the best of our knowledge, we believe that this study is important in the search for newer sources of bioactive compounds from the endophytic sources.

Materials and methods

Chemicals

All phenolic acids and flavonoid standards, trolox, ascorbic acid, ABTS [2, 2′-azino-bis(3- ethylbenzthiazoline-6-sulphonic acid)], DPPH (1, 1-diphenyl-2- picrylhydrazyl), and calf thymus DNA were purchased from Sigma-Aldrich (St. Louis, MO, USA). FolinCiocalteu's reagent was purchased from SRL Pvt. Ltd. (Mumbai, India). Solvents used for HPLC analyses were of HPLC grade. Sodium hypochlorite, potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride, sodium nitrite, aluminum chloride, potassium persulfate, all other general chemicals, and solvents were of analytical grade. Triple distilled water was used wherever necessary. Antibiotic disc and the Mueller-Hinton medium were purchased from Hi-media (Mumbai, India).

Collection of plant material

Polygonum chinense L. was collected from the Talacauvery subcluster (012° 17′ to 012° 27′ N and 075° 26′ to 075° 33′ E) of Western Ghats, in Kodagu district during June 2012 (Fig. 1). The plant parts (leaves and stem) were collected separately, placed in zip-lock polythene bags and brought and processed for isolation within 24 h of collection. A herbarium specimen has been deposited in the Botanical Survey of India (BSI), Western Range with the accession number 136243.

Isolation of endophytic fungi from plant parts

Endophytic fungal isolations was carried out under aseptic conditions according to Tejesvi et al. (2005). All plant parts were subjected to surface sterilization by soaking in 70% (*v*/*v*) ethanol for 1 min followed by sodium hypochlorite (3.5%) for 3 min and washed three–four times in sterile distilled water. Dried plant parts were cut into 1.0 cm × 0.1 cm × 0.1 cm

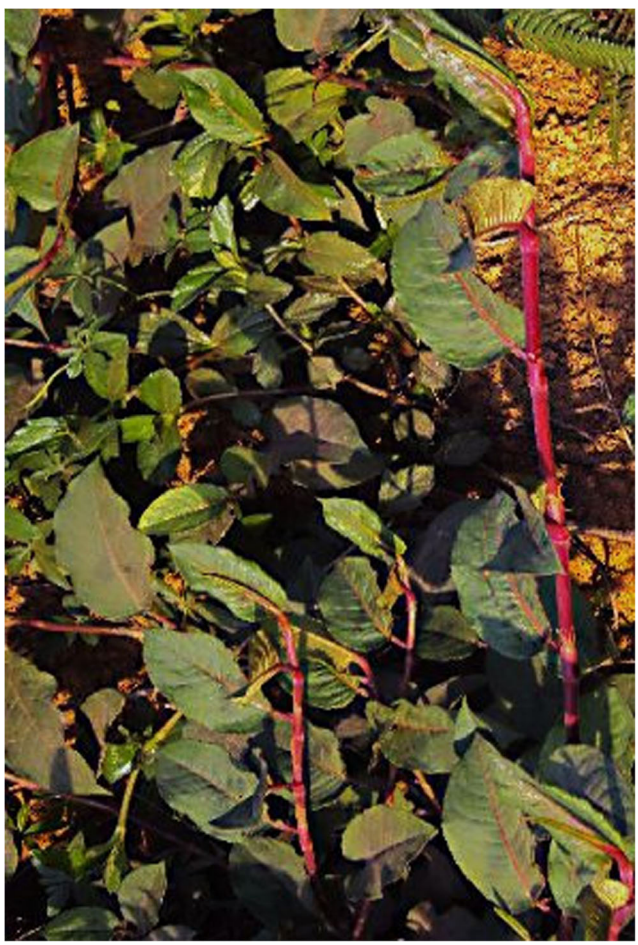

Fig. 1 *Polygonum chinense* L. collected from the Western Ghats of southern India

pieces under sterile conditions. A total of 400 fragments was plated on water agar media (2% *w*/*v*) for the isolation of endophytic fungi, supplemented with the antibiotic streptomycin (50 mg/l) to suppress bacterial growth. The effectiveness of the surface sterilization was confirmed by the disinfected plant segment imprint method on PDA (potato dextrose agar) plates from which no growth was observed (Schulz et al. 1998). The plates were sealed and incubated at 28 ± 2 °C with 12 h of light and dark cycles for 4–6 weeks. The plates were observed periodically. Fungal hyphae emerging from the plated fragments of tissues were cultured on PDA at 28 ± 2 °C for 10–15 days and maintained as the pure culture at 4 °C for further use.

Identification of endophytic fungi

Microscopic slides of each endophyte were prepared by staining with lactophenol cotton blue and examined under a light microscope (Labovision, India) for morphological analysis by the standard identification keys (Pitt 1988; Barnett and Hunter 1998; Leslie and Summerell 2006; Domsch et al. 2003). Based on the mycelial characteristics and spore structure ten representative endophytic fungi were selected for molecular identification and further analysis of their metabolites.

Molecular characterization by DNA sequence analysis of the ITS (internally transcribed spacer) region

Isolation of genomic DNA and amplification

Actively growing mycelial plugs from morphologically different endophytic fungi were inoculated into potato dextrose broth (PDB). The isolates were grown in still culture at 28 ± 2 °C for 7–10 days. The genomic DNA was extracted from the freeze-dried fungal mats by cetyltrimethylammonium bromide (CTAB) method with slight modifications (Ausubel et al. 1994). The DNA concentration was estimated by measuring the absorbance at 260 and 280 nm (Thermo Scientific Nanodrop 2000/2000c, Bangalore, India). Target regions of the rDNA ITS 1 and 2 regions and 5.8 rRNA gene were amplified using primers ITS 1 and ITS 4. The amplification was performed in a total reaction volume of 25 μL containing 200 μmol/L dNTP, 10 pmol/μL of each primer, and 50 ng template DNA. The amplification conditions consisted of an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of 92 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 10 min. The amplified product was subjected to sequencing at Chromous Biotech Pvt. Ltd. Bangalore, India. The endophyte sequences were aligned with the reference sequences using the BLAST algorithm and submitted to the NCBI GenBank nucleotide collection. The phylogenetic analysis of the endophytic strains was done by the alignment of multiple sequences with CLUSTAL W (Thompson et al. 1994) and a phylogenetic tree was constructed using default program.

Fermentation and extraction of metabolites

The pure culture of 10-day-old isolates was inoculated into PDB in duplicates and kept for incubation for 3 weeks at 28 ± 2 °C. The fermentation broth of each endophyte was extracted three times with ethyl acetate at room temperature and further concentrated by a Rotary flash evaporator (Superfit Model, PBU-6D, India). The residue obtained was designated as the crude dry extract and stored in glass vials, until use.

Detection of antibacterial activity

Test organisms

Two gram-positive bacteria viz. *Bacillus subtilis* (MTCC 121), *Staphylococcus aureus* (MTCC 7443) and four gram-negative bacteria viz. *Escherichia coli* (MTCC 729), *Pseudomonas aeruginosa* (MTCC 7093), *Enterobacter aerogenes* (MTCC 111), and *Klebsiella pneumoniae* (MTCC 661) were used. The test organisms were procured from the Department of Studies in Microbiology, University of Mysore, Karnataka, India.

Antibacterial activity

The inhibitory effect of the endophytic fungal extract was tested by disc diffusion method (Bauer et al. 1966). The crude extract of endophytic fungi (250 μg per disc) was dissolved in dimethyl sulfoxide (DMSO) and tested on Mueller-Hinton agar medium seeded with the test bacterium at (5 mm diameter, Whatman no. 1) concentration. Streptomycin (10 μg/disc) was used as positive control and the paper disc loaded with only DMSO as the negative control. The test plates were incubated for 24 h at 35 ± 2 °C and the inhibition zone was measured.

Determination of minimal inhibitory and minimum bactericidal concentration

The minimal inhibitory concentration (MIC) was determined by modifying the broth dilution method (Xu et al. 2008), using sterile 96-well microplate (Tarsons, Kolkata, India). The wells were filled with a reaction mixture containing 90 μl bacterial suspensions (10^6 cfu/ml) and 10 μl of the test sample with different concentrations (2 mg/ml to 0.02 mg/ml). The culture medium with 1% DMSO was used as the negative control and streptomycin sulfate (0.4 to 0.01 mg/ml) was a positive control. The microplates were incubated for 24 h at 35 ± 2 °C. After the incubation, 10 μl of the indicator 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)

(0.5 mg/ml phosphate buffer saline) was added to visualize the microbial growth. The lowest sample concentration at which no blue color appeared was determined as MIC. Wells containing MIC concentration and above were inoculated onto agar medium to check cell viability. The lowest concentration with no viable cells was determined as minimum bactericidal concentration (MBC).

Thin layer chromatography (TLC) and bio-autography

The ethyl acetate extract of endophytic fungi with antibacterial activity was subjected to TLC for the separation of the active compound. The solvent systems used as liquid phase was a mixture of chloroform: ethyl acetate: formic acid in the ratio 20:16:4. The developed chromatograms were subjected to agar overlay bio-autography (Rahalison et al. 1991).

Identification of antibacterial compounds through LC-MS analysis

Analyses of antimicrobial compounds by LC-electrospray ionization (ESI)–MS were carried out using Synapt G2 High definition mass spectroscopy (Waters, Milford, MA, USA). Liquid chromatography separation was performed on a reversed-phase Acquity UPLC BEH C_{18} column (2.1 × 50 mm) with a 1.7-μm particle size (dp) (Waters™, Milford, MA, USA). The LC condition was as follows: solvent A (0.1%) formic acid and solvent B (100%) acetonitrile. A gradient elution, 0–2.5 min 95% A, 5% B; 2.5–4.0 min 10% A, 90% B; 4.0–5.0 min 95% A, 5% B was used with a flow rate 0.7 ml/min. The UV detector was set to an absorbance wavelength of 280 to 340 nm. The LC elute was introduced directly into the ESI interface without flow splitting. The scan range of ESI-MS was *m/z* 100–1000. The drying gas (N2) flow was 500 L/h. The ESI capillary voltage was 3 kV ion mode.

GC-MS analysis of antibacterial compounds

GC-MS (GC-17A with QP5000 MS, Shimadzu Corp., Kyoto, Japan) was used to analyze the volatile and aliphatic compounds. An SPB-1 column (30 m × 0.32 mm with film thickness 0.25 μm; Sigma-Aldrich, St. Louis, MO, USA) was used and 2-μl samples dissolved in acetone was injected with split ratio 20:1. The analysis was carried out with oven temperature programmed at 50 °C (hold 3 min) and raised to 260 °C at a rate of 5 °C/min. The injection port temperature was 250 °C, transfer temperature was 200 °C and the ion source temperature was 180 °C. Helium was used as the carrier gas at a flow rate of 1 ml/min. The instrument was calibrated to scan range *m/z* 40–400. The compounds were identified by computer matching of their mass spectral fragmentation patterns with the NIST-MS library.

Determination of antioxidant capacity

DPPH radical scavenging activity

The quenching ability of DPPH was measured according to the procedure of Liu et al. (2007) with some modifications. A methanolic solution of DPPH (0.001 mM) was added to the fungal extract. The absorbance was read at 517 nm spectrophotometrically, after 20 min of incubation. The scavenging activity was expressed as IC_{50} (μg/ml). Ascorbic acid was used as the standard. The scavenging ability of the DPPH radical was calculated by the formula:

$$\%\text{scavenging} = \left[\left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{control}}\right] \times 100.$$

Reducing power assay

The reducing power was measured by the method of Oyaizu (1986) with some modifications. The fungal extracts were mixed with phosphate buffer (0.2 M, pH 6.5) and potassium ferricyanide (1%, 0.5 ml). The mixture was then incubated at 50 °C for 20 min. After incubation, TCA (10% *w/v*, 0.5 ml) was added and centrifuged at 3000 rpm for 10 min. To the supernatant, the same volume of distilled water and ferric chloride (0.1%, 300 μl) was added and the absorbance was measured at 700 nm. The activity was expressed as mg ascorbic acid (AA)/g dry extract.

DNA protection assay

DNA protection assay was conducted using calf thymus DNA (Lee et al. 2002). Calf thymus DNA (5 μg) was incubated with Fenton's reagent (30 mM H_2O_2, 50 mM ascorbic acid and 80 mM $FeCl_3$) and fungal extracts (150 μg/ml). The mixture was analyzed by 1% agarose gel electrophoresis after 30 min of incubation at 37 °C. The positive control reaction contained calf thymus DNA and Fenton's reagent. The negative control consisted of the calf thymus DNA. The results were documented using XR+ Molecular Imager Gel documentation system (Bio-Rad, USA).

Determination of total phenolic content

The total phenolic content of the fungal extracts was assessed according to the Folin–Ciocalteau (FC) method of Liu et al. (2007) with some modifications. One microliter of FC reagent and 2.0 ml of sodium carbonate (20%, *w/v*) was mixed with the crude extracts. The mixture was incubated for 45 min in the dark. The absorbance was read at 765 nm (T- 60, TTL Technology, India). The total phenolic content of the extracts was expressed as mg of gallic acid equivalent (GAE)/g of the extract.

Determination of total flavonoid content

The total flavonoid was determined according to the method of Barros et al. (2007). The fungal extract was mixed with sodium nitrite (5%, 75 μl). After 5 min aluminum chloride (10%, 150 μl) and sodium hydroxide (1 M, 500 μl) were added. The absorbance was measured at 510 nm. The content of flavonoid was calculated using a calibration curve of catechin and the results were expressed as mg of catechin equivalent (CE)/g of the extract.

Identification of antioxidative phenolics through HPLC analysis

Phenolic acids and flavonoids were separated by the reverse phase analytical HPLC using Shimadzu LC-8A (Shimadzu Corporation, Tokyo, Japan) HPLC fitted with C_{18} column (25 cm × 4 mm length, 5 μm, Kromasil, India) and a diode array detector. An isocratic mobile phase consisting of water:formic acid:acetonitrile:(78:2:20 *v*/*v*) was delivered at a flow rate of 1 ml/min to elute phenolic acids. The absorbance of phenolic acids was recorded at 280 nm. Flavonoids were eluted with a gradient of solvent A (water adjusted with acetic acid to pH 2.8) and solvent B (acetonitrile) as described by Das and Singh (2016). The gradient was linear to 10% B in 5 min, 23% B in 31 min, and 35% B in 43 min. The column was washed with 100% B for 6 min and equilibrated for 6 min at 100% A to start the next sample. The absorbance was recorded at 260 nm. The peaks were identified by comparison with the standards. The HPLC water was purified by a Milli-Q System. The LC fragments of all individual peaks were collected in separate vials and used for ESI-MS/MS analysis.

ESI-MS/MS analysis of antioxidative phenolics

ESI-MS and MS/MS were performed using quadrupole time of flight (Q-TOF) mass spectrometer (Micromass Waters, Milford, MA, USA) with electrospray ionization (ESI). The instrument was calibrated through a mass range of 100–1000 and operated at negative mode $[M\text{-}H]^-$. The capillary voltage was 3 kV; source and desolvation temperatures were 120 and 300 °C, respectively; cone gas (argon) and desolvation gas (nitrogen) flow rates were 50 L/h and 500 L/h, respectively. MS/MS spectra were acquired using collision energy of 20 V. The *m*/*z* ratio, as well as fragmentation patterns, was used for the confirmation of the phenolic acids and flavonoids.

Data and statistical analysis

The colonization frequency (CF) was calculated by the following formula: $\%\ CF = [N_{col}/N_t] \times 100$, where, N_{col} is the number of tissue segments colonized by a fungus; N_t is a total number of tissue segments plated (Nalini et al. 2014).

Data reported as the mean ± standard deviation (SD) of three independent replicates. Comparison of means was analyzed with one-way ANOVA and Tukey-Kramer multiple comparisons tests using Graph Pad InStat 3.0. Any two data were considered statistically significant at $p < 0.05$ and denoted by different superscripts.

Results

Isolation and identification of fungal endophytes

Endophytic fungi were isolated from the stem and leaf parts of *P. chinense* L. A total of 264 isolates distributed in 10 endophytic species were recovered from 400 plant fragments. The isolates were identified with their spore morphology as well as by analyzing the DNA sequence of the ITS region. The identified strains with their GenBank accession numbers, isolation number and colonization frequency (%CF) is depicted in Table 1. The result indicated that the stem part had number of isolates (141) than leaves (123). The ten endophytic species belonged to seven genera. Three different species of *Fusarium* and two of *Alternaria* were recovered. *Fusarium solani* showed the highest %CF (15.9) followed by *Fusarium chlamydosporum* (15.2). The least %CF was recorded for *Alternaria alternata*. The phylogenetic relationships of isolated endophytic strains were deduced by constructing a phylogenetic tree using ITS sequences (Fig. 2). The isolates were clustered into five major clades, each of which was further divided into subclades except the clade of *Cladosporium tenuissimum*.

Antibacterial activity

The antibacterial activity of strains was tested against six pathogenic bacteria and the appeared area of inhibition zone is presented in Table 2. The strains were further tested for MIC and MBC. The results are depicted in Table 3. *Penicillium canescens* (Fig. 3) exhibited the highest inhibition zone against all the pathogens except *B. subtilis* followed by *F. chlamydosporum*. The inhibition zone formed by *P. canescens* is represented in Fig. 4. *Bacillus subtilis* was found to be resistant against all the extracts. *Alternaria longipes* did not show any activity. *Curvularia geniculata* did not depict any inhibition zone at 250 μg but showed inhibition at higher concentration against a gram-positive (*P. aeruginosa*) and a gram-negative (*E. coli*) bacteria. All the endophytic fungal extracts showed inhibitory activity against *E. coli*, the concentration ranged from 2 to 0.06 mg/ml. *Emericella nidulans* showed an inhibition zone only against *E. coli*.

Penicillium canescens showed good antibacterial activity against all the pathogens. Therefore, the extract of *P.*

Table 1 Taxonomic identification and percent colonization frequency (%CF) of the fungal endophytes isolated from *P. chinense*

Endophytic fungi	Code	Accession no.	Leaf*		Stem*		Total %CF
			I	%CF	*I*	%CF	
Bipolaris sorokiniana	PC-WG-01	KY024401	-	-	11	5.5	2.8
Penicillum canescens	PC-WG-02	KY052774	-	-	14	7.0	3.6
Fusarium chlamydosporum	PC-WG-03	KY072925	27	13.5	34	17.0	15.2
Cladosporium tenuissimum	PC-WG-04	KY039168	-	-	27	13.5	6.9
Fusarium solani	PC-WG-05	KY039169	25	12.5	39	19.5	15.9
Alternaria alternata	PC-WG-06	KY039171	11	5.5	-	-	2.5
Curvularia geniculata	PC-WG-07	KY052771	26	13.0	-	-	6.2
Fusarium incarnatum	PC-WG-08	KY052774	5	2.5	7	3.5	2.8
Emericella nidulans	PC-WG-10	KY039167	16	8.0	-	-	4.1
Alternaria longipes	PC-WG-11	KY039170	22	11	9	4.5	7.7

*200 fragments were plated from leaf and stem fragments respectively. *I* number of isolates. "-" indicates the absence of the endophytic fungi in the respective plant part

canescens was further characterized for the identification of antimicrobial compounds by TLC and bio-autography.

TLC and bio-autography

The presence of antimicrobial compounds in *P. canescens* extract was detected by TLC and bio-autography methods. This aided direct visualization of an active compound with an inhibition zone at Rf − 0.88 on the chromatogram (Fig. 5a). The antibacterial activity was confirmed by the formation of halo around the chromatogram post-spray with 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Fig. 5b), the reliable indicator cellular metabolic activity. The compound was collected carefully and further characterized by liquid chromatography (LC), gas chromatography (GC), and mass spectral analysis.

Identification of antimicrobial compounds through LC-MS and GC-MS analysis

The identification of phenolic compound was done by LC-MS, while GC-MS was employed to identify volatile and aliphatic compounds. LC-MS result revealed the presence of quercetin in the TLC fraction of the extracts. GC-MS of the TLC fraction revealed four peaks corresponding to the presence of 1,7-dimethyl-4-(1-methyl ethyl) cyclodecane (germacrene), phthalic acid ester, Cyclopentane, 1,1,3,3-tetramethyl-and 1-Nitrododecane (Table 4). The chromatogram of liquid and gas chromatography with mass spectra is represented in Fig. 6.

Antioxidant capacity

DPPH radical scavenging capacity

The DPPH radical is almost stable and extensively used for the antioxidant activity. The radical scavenging activity is presented as 50% scavenging activity (IC_{50}) in Table 5. The results showed that the IC_{50} value of the fungal extracts varied from 73.6 ± 3.3 to 1114.9 ± 71.1 μg/ml. *Penicillium canescens* showed the highest scavenging activity with 73.6 ± 3.3 μg/ml, followed by *F. chlamydosporum* (126.8 ± 6.7 μg/ml). *Bipolaris sorokiniana* and *A. alternata* exhibited the IC_{50} value of 319.2 ± 40.2 and 366.2 ± 15.8 μg/ml respectively.

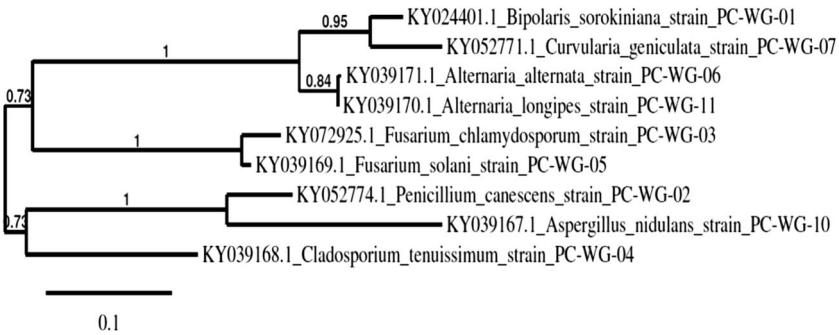

Fig. 2 Phylogenetic tree based on ITS sequences of the endophytic fungal isolates. The left side indicates the sequence of the nearest strain. The scale bar indicates 0.1 substitutions per nucleotide position

Table 2 Antibacterial activity of fungal endophytes isolated from *P. chinense* against six pathogenic bacteria

Fungal strains extract	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Staphylococcus aureus*	*Escherichia coli*	*Enterobacter aerogenes*	*Klebsiella pneumoniae*
P. canescens	19.5 ± 0.9	-	18.6 ± 1.0	21.9 ± 2.1	23.4 ± 0.7	15 ± 1.1
F. chlamydosporum	12.8 ± 1.1	-	14.0 ± 0.5	16.8 ± 0.6	21.2 ± 0.1	13.5 ± 0.9
A. alternata	11.3 ± 0.2	-	9.9 ± 0.3	13.8 ± 0.3	8.9 ± 0.2	11.2 ± 0.1
B. sorokiniana	14.4 ± 1.3	-	10.5 ± 0.4	12.2 ± 1.4	9.2 ± 0.1	10.2 ± 0.5
F. solani	14.6 ± 0.2	-	10.6 ± 0.4	11.6 ± 1.0	-	11.7 ± 0.5
C. geniculata	-	-	-	-	-	-
C. tenuissimum	11.7 ± 0.9	-	9.5 ± 0.7	-	-	10.5 ± 0.2
E. nidulans	-	-	-	7.3 ± 0.1	-	-
F. incarnatum	-	-	-	9.0 ± 0.1	8.5	8.3 ± 0.3
A. longipes	-	-	-	-	-	-
*Streptomycin**	33 ± 0.3	32 ± 0.1	31.5 ± 0.5	20 ± 0.1	22 ± 0.1	30 ± 0.2

Data are reported as mean ± SD of three independent analyses ($n = 3$). *Streptomycin 10 μg disc used; "-" indicates the absence of inhibition zone by the endophytic fungal extract in the disc diffusion method

Reducing power assay

The reducing power of ferric ion to ferrous ion by the fungal extracts is represented in terms of ascorbic acid equivalent (Table 5). The values of reducing power assay ranged from 12.8 ± 0.2 to 95.8 ± 2.5 mg AA/g dry extract. *Penicillium canescens* showed the highest activity (95.8 ± 2.5 mg AA/g dry extract) followed by *F. chlamydosporum* (84.7 ± 2.1 mg AA/g dry extract) and *A. alternata* (48.2 ± 2.0 mg AA/g dry extract).

DNA protection assay

Among the five isolates, *P. canescens* extract visibly showed the DNA protection ability by inhibiting its fragmentation through the scavenging of –OH radicals generated by Fenton's reagent (Fig. 7).

Among the endophytic fungal strains, *P. canescens* and *F. chlamydosporum* exhibited high total phenolic content and antioxidative capacities. Hence the phenolic compounds present in the crude extracts were further characterized by HPLC and ESI-MS/MS techniques.

Phenolic and flavonoid content

The total phenolic content (TPC) of the extracts is represented in Table 5. TPC of the extracts ranged from 9.9 ± 0.7 to 78.9 ± 2.5 mg GAE/g dry extract. *Penicillium canescens* extract showed the highest total phenolic content (78.9 ± 2.5 mg GAE/g dry extract) followed by *F. chlamydosporum* extracts (50.3 ± 0.5 GAE/g dry extract respectively). *A. longipes* exhibited the least phenolic content (9.9 ± 0.7 5 mg GAE/g dry extract).

Table 3 Minimal inhibitory (MIC mg/ml) concentration and minimum bactericidal concentration (MBC mg/ml) of fungal endophytes

Endophytic fungal strain extract	*P. aeruginosa*		*S. aureus*		*E. coli*		*E. aerogenes*		*K. pneumoniae*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
P. canescens	0.1	0.12	0.1	0.12	0.08	0.1	0.06	0.08	0.14	0.16
F. chlamydosporum	0.14	0.16	0.18	0.2	0.12	0.14	0.08	0.1	0.18	0.2
A. alternata	0.18	0.2	0.2	0.4	0.14	0.16	0.4	0.6	0.18	0.2
B. sorokiniana	0.12	0.14	0.18	0.2	0.18	0.2	0.4	0.6	0.2	0.2
F. solani	0.18	0.2	0.18	0.18	0.18	0.2	-	-	0.16	0.18
C. geniculata	1.8	2	-	-	1.6	1.6	-	-	-	-
C. tenuissimum	0.16	0.18	0.4	0.6	1.4	1.6	-	-	0.2	0.4
E. nidulans	-	-	-	-	1.2	1.4	-	-	2.0	-
F. incarnatum	2.0	-	-	-	0.4	0.6	0.6	0.8	0.6	0.8
A. longipes	-	-	-	-	2	-	-	-	-	-

"-" indicates the absence of inhibition of endophytic fungal extracts in the MIC and MBC assay

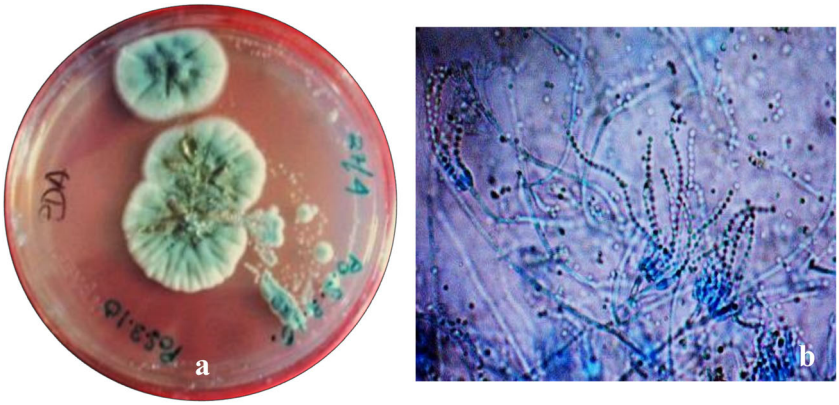

Fig. 3 *Penicillium canescens* isolated as an endophyte from the stem of *P. chinense* L. **a** Velutinous colonies on PDA medium **b**. Light microscopy view (× 40 magnification) with biverticillate branching conidiophores with phialides and metulae bearing chains of globose roughened conidia

Flavonoid was detected in seven endophytic strains (Table 5). The total flavonoid content (TFC) ranged from 5.9 ± 0.4 to 33.6 ± 1.0 mg CE/g dry extract. *Penicillium canescens* exhibited high flavonoid content (33.6 ± 1.0 mg CE/g dry extract). TFC of *B. sorokiniana* and *A. alternata* was documented as 17.5 ± 0.5 and 14.6 ± 0.2 mg CE/g dry extract respectively. The lowest TFC was recorded for *A. longipes*. Flavonoid was not detected in *C. geniculata*, *Cladosporium tenuissimum*, and *Fusarium incarnatum* extracts.

Identification of antioxidative phenolics through HPLC and ESI-MS/MS analysis

The extracts of *P. canescens* and *F. chlamydosporum* were characterized for the phenolic compounds. HPLC analysis revealed that *P. canescens* extract contained phenolic acids as well as flavonoids whereas, *F. chlamydosporum* contained solely phenolic acids (Fig. 8). The compounds were isolated individually and subjected to ESI-MS and MS/MS analyses for the confirmation of their presence. The concentrations of

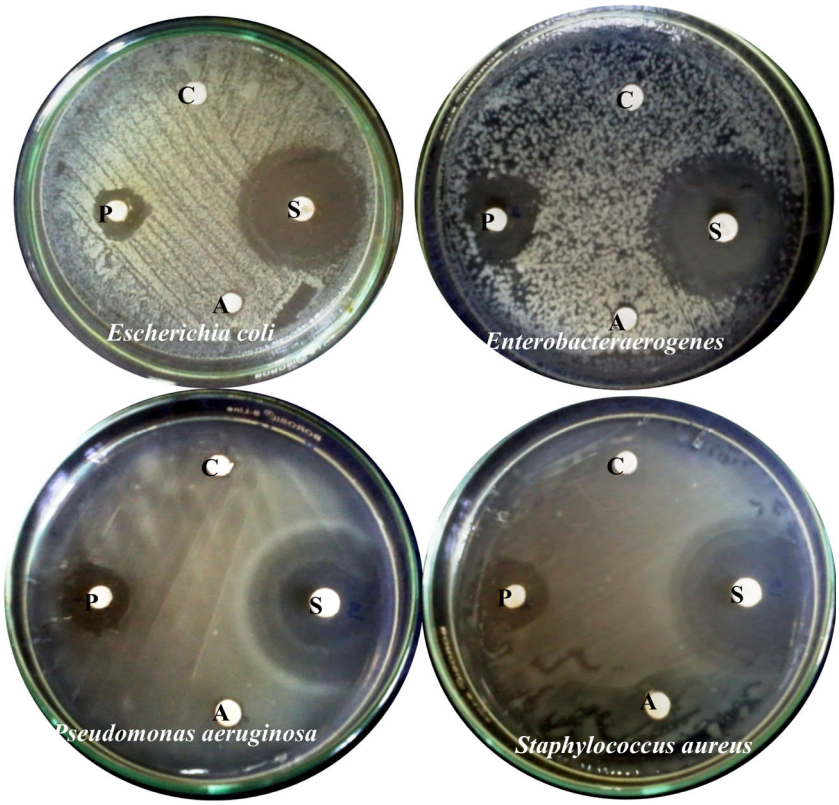

Fig. 4 Inhibition zone formed by *P. canescens* extract. *Penicillium canescens* extract (250 μg/ml) was tested for the antibacterial potential against the test bacteria seeded onto Mueller-Hinton agar (MH) by the disc diffusion assay. The plates were incubated for 24 h at 35+ °C and the presence of or absence of the inhibitory zone around the disc was measured and represented. S streptomycin disc, C negative control, P *P. canescens* extract, A *A. longipes* extract

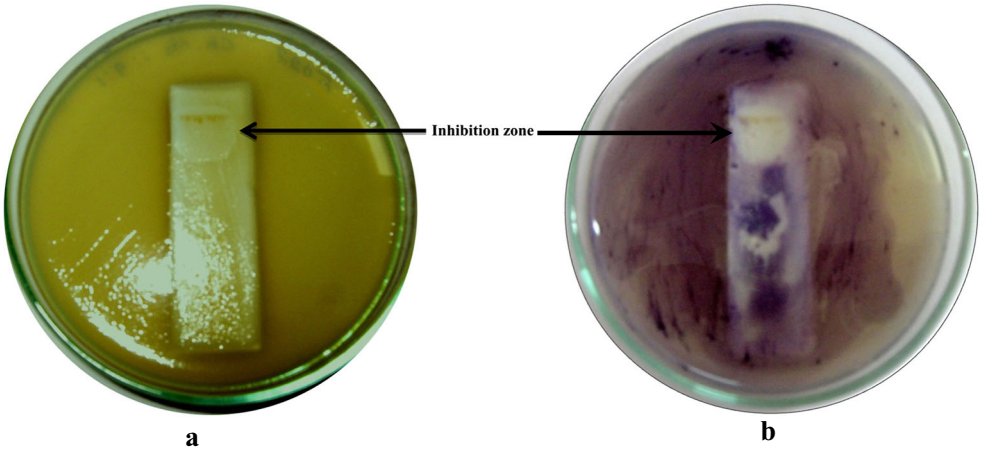

Fig. 5 Bio-autography of *P. canescens* extract. *Penicillium canescens* extract was subjected to TLC. The chromatogram was placed on MH agar plate and molten agar seeded with the bacterial suspension of 1.5×10^8 cfu/ml was poured on the chromatogram. Post-solidification, the plates were place at 4 °C for 3 h and observed for the halo around the chromatogram (**a**). The antibacterial activity was confirmed by the formation of halo around the chromatogram post-spray with MTT (**b**)

individual phenolic compounds in both the extracts and MS/MS fragmentation patterns are depicted in are presented in Table 6. Caffeic acid (CA) was found in both the extracts. Vanillic acid (VA) was present only in *P. canescens*, whereas ferulic (FA) in *F. chlamydosporum. Penicillium canescens* also contained a flavonoid compound, quercetin (6.9 ± 0.3 mg/of extract).

The peak at the retention time (RT) 5.3 ± 0.3 min with a major ion 179 *m/z* and a fragment of 135 *m/z* [M-H-44] due to the loss of a –COO group, i.e., decarboxylation was confirmed as CA. The peak at RT 6.0 ± 0.3 min had a major ion of 167 *m/z* and a fragment of 152 *m/z* due to the loss of a CH_3 moiety, i.e., demethylation [M-H-15] on the aromatic benzene ring, thus confirmed as VA. FA was confirmed at RT 11.3 ± 0.1 with major ion of 193 *m/z* and 3 fragments of 178 *m/z* (demethylation), 149 *m/z* (decarboxylation) and 134 *m/z* (both demethylation and decarboxylation). The flavonoid molecule quercetin was identified at RT 42.8 ± 3.1, produced fragments of 151 and 179 *m/z* as a result of cleavage of the heterocyclic C-ring by Retro-Diels-Alder rearrangement (Sun et al. 2007).

Discussion

Polygonum chinense L. is a medicinal plant, found growing in the Western Ghats region of India. Being a biodiversity hotspot, this region attracts researchers to unfold the treasure house of mother nature, causing irreparable damage to biodiversity. Many researchers in the recent past have explored the richness of endophytes instead of using the plants per se (Nalini et al. 2014; Uzmaa et al. 2016; Das et al. 2017). The endophytes are rich sources of bioactive compounds. Hence, the present study deals with the isolation of endophytic fungi and their potential bioactive compounds. A total of ten endophytic fungal strains distributed in seven genera viz., *Fusarium*, *Alternaria*, *Penicillium*, *Curvularia*, *Emericella, Cladosporium*, and *Bipolaris* were identified. In order to identify the fungal strains to the species level, both morphological and phylogenetic analyses using molecular tools were used. The taxonomic identification of dark septate endophytes is difficult due to the lack of clearly defined sexual stages, the absence of asexual spores and lack of identifiable morphological traits (Samaga and Rai 2016). Hence, sequence analysis of the strains is essential. The blast result of ITS region showed 98–100% similarity and almost all the fungi were previously reported as endophytes. *Polygonum chinense* was previously reported to host 15 endophytic fungal taxa with *Fusarium* exhibiting high fungal diversity (Huang et al. 2008a). In the present study, ten morphologically distinct taxa with high colonization frequency was recorded for two *Fusarium* species, which again substantiates the dominance of *Fusarium.* Apart from *Fusarium*, *A. longipes* recorded high CF (7.7%) while other species of *Alternaria* showed lowest CF (2.5%). *Alternaria* sp. were previously isolated as common endophytic fungi from tropical, subtropical, and temperate regions (Guo et al. 2004; Jordaan et al. 2006; Aly et al. 2007). In

Table 4 Compounds identified through GC-MS from *P. canescens* extract

Peak	Retention time (min)	Molecular weight	Chemical structure	Compound name	% area
1	27.09	126	C_9H_{18}	Cyclopentane, 1,1,3,3-tetramethyl-	1.507
2	30.79	306	$C_{18}H_{26}O_4$	Phthalic acid, butyl hexyl ester	0.909
3	31.1	210	$C_{15}H_{30}$	1,7-Dimethyl-4-(1-methyl ethyl)cyclodecane	1.36
4	34.77	215	$C_{12}H_{25}NO_2$	1-Nitrododecane	0.679

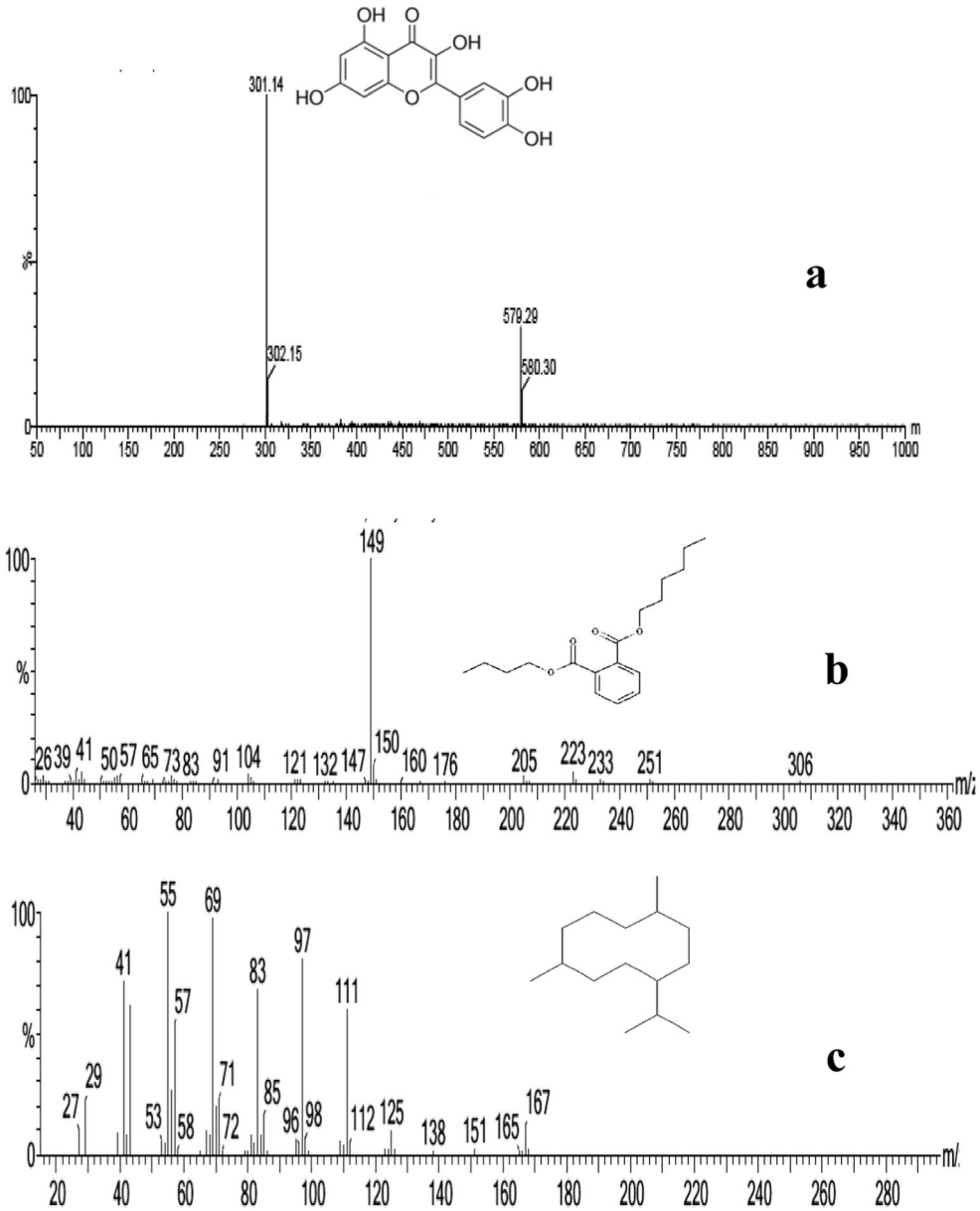

Fig. 6 Mass spectra of the antibacterial compound. **a** Quercetin. **b** Phthalic acid, butyl hexyl ester. **c** Germacrene [1,7-dimethyl-4-(1-methyl ethyl) cyclodecane]

addition, Aly et al. (2008) reported an endophytic *Alternaria* sp. from another species *Polygonum* (*P. senegalense*). The differences in the diverse endophytic taxa isolated from *P. chinense* sampled from distinct regions can be attributed to the geographical location of the plant.

A variety of relationships exist between the fungal endophytes and its host plant. It can be symbiotic, mutualistic, antagonistic, or slightly pathogenic (Huang et al. 2008a). Due to this interaction with the host plant, the endophytic fungi can produce a plethora of substances for potential use in modern medicine, agriculture, and industry (Mitchell et al. 2008). The endophytes are synergistic to their host. The endophytes, against their nutritional benefits, release metabolic substances beneficial to the host plant in resisting the abiotic stresses and attacks from herbivore pathogens and insects. The array of metabolites synthesized by the endophytes confers plants with more resistance. Plants that inhabit specific endophytes are often able to grow faster due to the production of phytohormones enabling them to dominate in a particular environment (Kaul et al. 2012).

Polygonum chinense is traditionally used to treat various diseases. Huang et al. (2008b) reported the plant extract to exhibit strong antibacterial activity against *Bacillus cereus* (MIC, 2.50 mg/ml), *Listeria monocytogenes* (MIC, 5.00 mg/ml), and *S. aureus* (MIC, 5.00 mg/ml). Maharajan et al. (2012) reported that *P. chinense* extract possess strong antimicrobial activity against *B. subtilis*, *S. aureus*, *P. aeruginosa*, *E. coli*, and *Aspergillus niger*. In the present study, the isolated endophytes were tested for the antibacterial activity, where 80% of them showed antibacterial potency against one or more

Table 5 Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant capacity of fungal endophytes from *P. chinense*

Fungal strains/ extracts	TPC (mg GAE/g dry extract)	TFC (mg CE/g dry extract)	Reducing power (mg AA/g dry extract)	DPPH radical scavenging capacity (IC_{50} μg/mL)
P. canescens	78.9 ± 2.5^{a}	33.6 ± 1.0^{a}	95.8 ± 2.5^{a}	73.6 ± 3.3^{b}
F. chlamydosporum	50.3 ± 0.5^{b}	12.5 ± 0.5^{d}	84.7 ± 2.1^{b}	126.8 ± 6.7^{c}
A. alternata	24.9 ± 1.1^{c}	14.6 ± 0.2^{c}	48.2 ± 2.0^{c}	319.2 ± 40.2^{d}
B. sorokiniana	22.7 ± 0.8^{d}	17.5 ± 0.5^{b}	44.3 ± 0.8^{d}	366.2 ± 15.8^{d}
F. solani	$21.4 \pm 1.9d^{e}$	10.1 ± 0.1^{e}	40.0 ± 1.1^{e}	440.5 ± 40.8^{e}
C. geniculata	19.3 ± 0.8^{e}	-	37.6 ± 1.1^{e}	472.5 ± 8.7^{e}
C. tenuissimum	17.8 ± 1.6^{f}	-	25.6 ± 0.8^{g}	542.6 ± 44.0^{f}
E. nidulans	16.5 ± 0.9^{f}	7.5 ± 0.3^{f}	30.1 ± 1.1^{f}	581.1 ± 35.2^{f}
F. incarnatum	13.6 ± 0.6^{g}	-	18.8 ± 1.0^{h}	766.9 ± 33.1^{g}
A. longipes	9.9 ± 0.7^{h}	5.9 ± 0.4^{g}	12.8 ± 0.2^{i}	1114.9 ± 71.1^{h}
			Ascorbic acid	7.7 ± 0.02^{a}

Data are reported as mean ± SD of three independent analyses ($n = 3$). Mean with the different superscript within a column are significantly different ($p < 0.05$) by one-way ANOVA test. "-" indicates the absence of activity in the respective assays by the endophytic fungal extracts

pathogenic bacteria. Unlike plant extracts, the endophytes demonstrated activity against *B. subtilis*. *Penicillium canescens* extract exhibited strong antibacterial activity against *E. coli* (MIC, 0.06 mg/ml), *P. aeruginosa* (MIC, 0.08 mg/ml), *S. aureus* (MIC, 0.1 mg/ml), and *K. pneumoniae* (MIC, 0.1 mg/ml). *Fusarium chlamydosporum*, *A. alternata*, and *B. sorokiniana* also showed antibacterial potency against all pathogens employed except *B. subtilis* (MIC, 0.2–0.08 mg/ml). The results clearly showed that the endophytes are mightier antibacterial agents than their host plant.

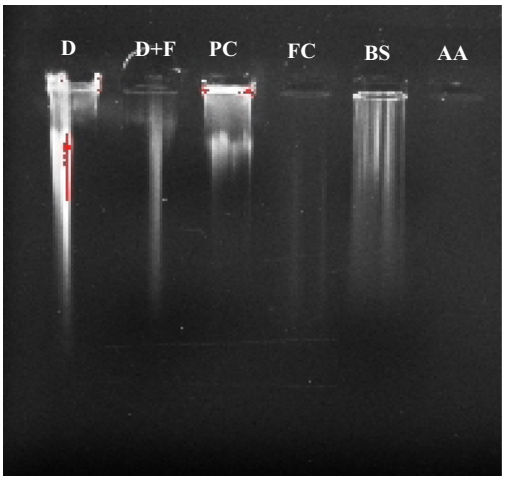

Fig. 7 DNA protection assay of endophytic fungal extracts. Calf thymus DNA (5 μg) was incubated with Fenton's reagent and the endophytic fungal extracts (150 μg/ml). After the incubation at 37 °C for 30 min, the mixture was run through agarose gel (1%) electrophoresis. The results were documented using XR+ Molecular Imager Gel documentation system (Bio-Rad, USA). D calf thymus DNA, (D + F) DNA + Fenton's reagent, PC *P. canescens*, FC *F. chlamydosporum*, BS *B. sorokiniana*, AA *A. alternata*

The endophytic *P. canescens* was previously reported to exhibit antibacterial activity against *E. coli*, *B. subtilis*, and *S. aureus* (Malhadas et al. 2017) without determining the MIC of the extract. In order to determine the exact potency of the extract, the MIC is an important parameter to perform and the present study demonstrates the same.

A literature survey by Yu et al. (2010) reveals species of *Penicillium* produce antimicrobial compounds. However, most authors have reported the activities of endophytic *P. canescens* against phytopathogenic fungi (Bertinetti et al. 2009; Nicoletti et al. 2014). In the present study, we have attempted the characterization of compounds responsible for antibacterial activities. Since *P. canescens* showed strong activity against the bacterial pathogens, the active compounds of this endophyte were isolated through bioautography and identified through LC-MS and GC-MS. A flavonoid compound-quercetin along with germacrene and phthalic acid ester (1,2-benzene dicarboxylic acid, butyl hexyl ester) were identified. Endophytes are chemical synthesizers inside plants (Owen and Hundley 2004). In other words, they play a role as a selection system for microbes to produce low toxic bioactive substances for higher organisms (Strobel 2003). Alvin et al. (2014) reported that endophytes are able to produce a similar secondary metabolite as their host. In previous studies, the plant extract of *P. chinense* was also found to contain quercetin-3-glucoside and quercetin-3-galactoside (Huang et al. 2008b). Quercetin has previously been reported to exert antibacterial activity against *S. aureus* and *S. epidermidis* (Hirai et al. 2010). The plasticizer compound 1,2-benzene dicarboxylic acid ester has also been reported from *P. chinense* plant by GC-MS analysis (Ezhilan and Neelamegam 2012). Both

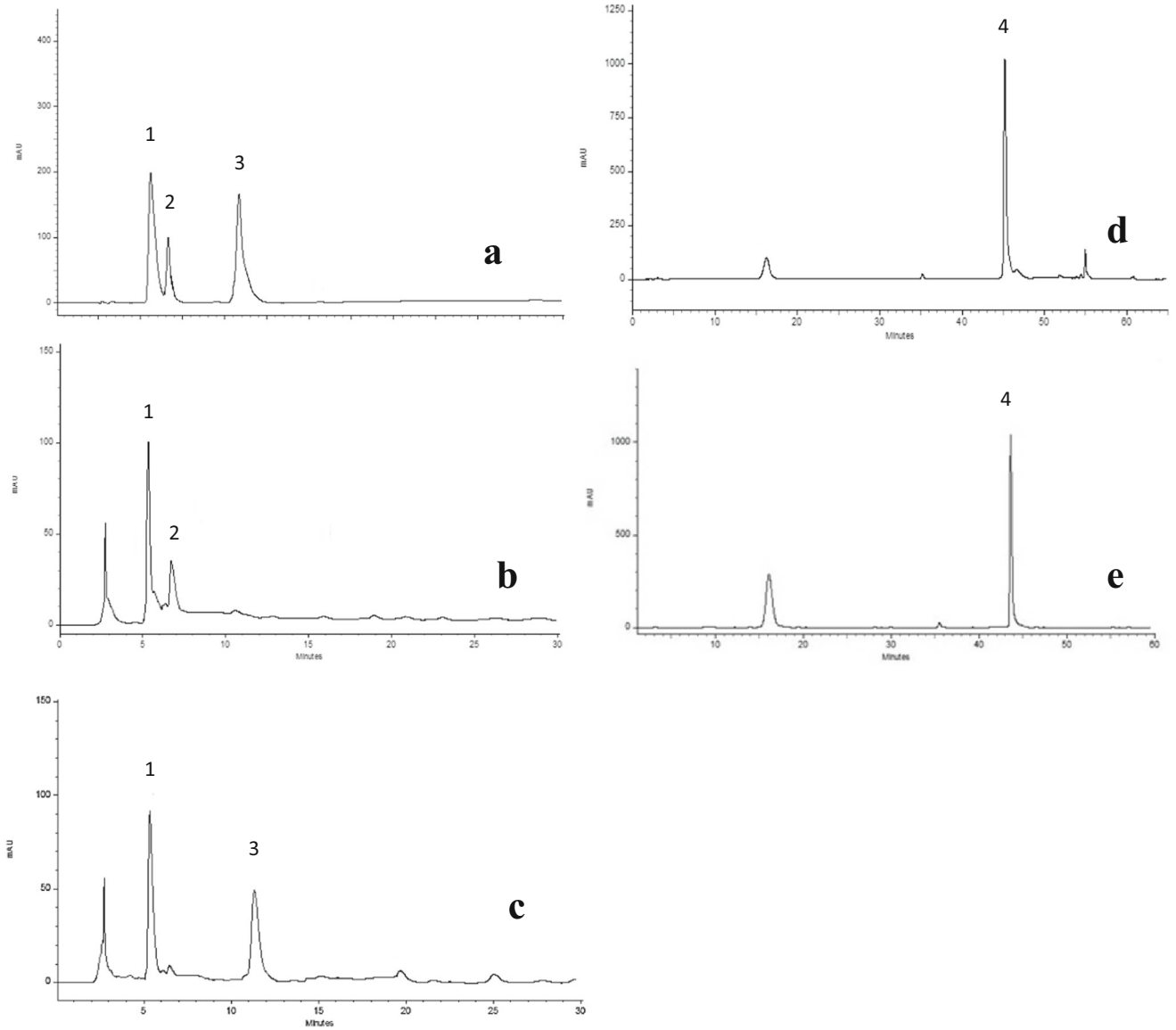

Fig. 8 HPLC chromatograms of phenolic compounds. **a** Separation of phenolic acids in the standard mixture (at 280 nm). **b** *P. canescens*. **c** *F. chlamydosporum*. **d** Separation of quercetin in a standard mixture (at 260 nm). **e** *P. canescens*. 1 caffeic acid, 2 vanillic acid, 3 ferulic acid, 4 quercetin

bioactive compounds are produced by the endophytic *P. canescens*, isolated from *P. chinense* in the present study. These pieces of evidence substantiate endophytes as alternative sources of plant metabolites. Phthalic acid ester

Table 6 Concentration and ESI-MS/MS analysis of phenolic compounds of endophytic fungal extracts

Source fungal strain	RT of peak	*m/z* [M-H]$^-$	MS/MS fragment	Compound identified	Concentration (mg/g of extract)
P. canescens	5.3 ± 0.3	179	134	Caffeic acid	4.3 ± 0.1
F. chlamydosporum	5.5 ± 0.3	179	134	Caffeic acid	3.3 ± 0.02
P. canescens	6.3 ± 0.3	167	152, 123	Vanillic acid	2.7 ± 0.07
F. chlamydosporum	11.3 ± 0.1	193	178, 149, and 134	Ferulic acid	0.95 ± 0.001
P. canescens	42.8 ± 3.1	300	151, 179	Quercetin	6.9 ± 0.3

Data are reported as mean ± SD of two independent analyses ($n = 2$)

reported in the present study was found to be antimicrobial by previous researchers (Barakat and Beltagy 2015). In addition, Al-Bari et al. (2006) isolated phthalate derivative from *Streptomyces bangladeshiensis* and reported to have good potency against *B. subtilis* and *S. aureus*, whereas Uddin et al. (2013) reported the compound to possess antiviral activity against dengue and chikungunya viruses.

Germacrene identified in this study is a sesquiterpene. Germacrene B and germacrene D are previously reported from *Guatteria australis* leaf-essential-oil showed antibacterial activity against *S. aureus* and *E. coli* (MIC 250 mg/ml) and demonstrated antioxidant activity (Siqueira et al. 2015). Terpenes also have unique antioxidant activity in their interaction with free radicals (Dillard and German 2000). *Penicillium canescens* is reported to have antioxidant capacity against DPPH radical scavenging (IC_{50}73.6 ± 3.3 μg/ml) and reducing ability of Fe^{3+} to Fe^{2+}. The antioxidative capacity is consistent with the total phenolic content.

The oxidative stress causes DNA damage and interruption of the cell cycle (Pizarro et al. 2009) which can lead to other chronic diseases. *Penicillium canescens* alone exhibited DNA protection ability against –OH-induced DNA damage and was ascertained to be a potential DNA protective agent.

Flavonoid was detected in seven endophytic strains among the ten strains. The extract of *P. canescens* exhibited highest TFC (33.6 ± 1.0 mg CE/g). Flavonoids were reported as important bioactive constituents of *Polygonum* species (Huang et al. 2008b) hence rationalizes the presence of high flavonoid content in endophytes.

HPLC analysis of *P. canescens* and *F. chlamydosporum* extracts revealed that these isolates contained various phenolic acids. The presence of bulky side chains (Jing et al. 2012) and the number of hydroxyl moieties attached to the aromatic ring of the benzoic or cinnamic acid molecules (Karamac et al. 2005) was favorable for the DPPH radical scavenging activity of phenolic acids. Both the extracts contained CA that have two –OH groups and a bulky side chain (CH=CHCOOH) may be responsible for the high scavenging activity of the extracts. Other than CA, *P. canescens* contained VA (2.7 ± 0.07 mg/g of extract) and *F. chlamydosporum* contained FA (0.95 ± 0.001 mg/g of extract). In addition, *P. canescens* extract also contained quercetin (6.9 ± 0.3 mg/g of extract) which augments its antioxidative capacity. Quercetin present in plant extracts of the genus *Polygonum* (Huang et al. 2008a, 2008b) possesses anti-inflammatory and free radical scavenging activity (Muzitano et al. 2006). The presence of quercetin also signifies the DNA protective capacity of *P. canescens* as the compound was reported to inhibit H_2O_2-induced (Duthie et al. 1997) and cadmium-induced DNA damage (Çelik and Arinç 2010).

Conclusions

In order to cater to the need of constant development of novel antibiotics against the drug-resistant pathogens, the studies on bio-prospecting while selecting the most suitable sources are keys to success. The Western Ghats of India, popularly known as biodiversity hotspot, is a source of many medicinal plants which are yet to be explored. Based on the background that many plant bioactive compounds are actually produced by their microbial symbionts, exploration of the endophytes from these medicinal plants will assist in isolating and producing their active components. The present study explores various fungi isolated as endophytes from the plant *P. chinense*. To the best of our knowledge, these endophytes were not fully profiled for their bioactive metabolites. The strain, *P. canescens* was found to have high antibacterial properties against gram-positive and gram-negative pathogens as well as significant antioxidative capacities. The strain *F. chlamydosporum*, displayed significant anti-radical and ferric reducing capacities. The secondary metabolites characterized by chromatography and mass spectroscopy revealed the presence of germacrene, phthalic acid esters, short-chain hydrocarbons, and phenols. The bioactivities of these compounds are well recognized in the literature. Hence, our findings encourage the exploration of these fungi for bioprospecting in search of novel compounds which are safe and efficacious for human use. Therefore, the potential of drug discovery using endophytes from traditional medicinal plants is immense.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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