



Endophytic fungi associated with *Psychotria flavida* Talbot., an endemic plant of Western Ghats (India) and their bioactive potential

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Abstract

Psychotria flavida Talbot. an endemic plant of Western Ghats was explored for endophytic mycoflora. The endophytic fungi isolated from leaf and stem were identified with the aid of morphology and molecular methods. *Lasiodiplodia theobromae* and *Picchia guillermondii* were commonly present in both the sites. Overall colonization frequency of leaf samples was found to be higher than stem samples in both the places. Studies on seasonal variation of the occurrence of these fungi indicated a higher frequency during winter as compared to monsoon and summer. Further, endophytic fungi were verified for bioactive diagnosis using in vitro antimicrobial (disc diffusion method) and antioxidant (DPPH free radical scavenging). Among the 15 crude extracts of different endophytes tested, the extracts of *Lasiodiplodia theobromae* possessed potentially better antibacterial and antioxidant activity. The present study divulges that the endophytic fungi *L. theobromae* could be a potential source of antimicrobial and antioxidant drugs.

Keywords *Psychotria flavida* · *Lasiodiplodia theobromae* · Antibacterial · Antioxidant

Introduction

Endophytes are the microorganisms that reside in the tissues of living plants at any stage of their life cycle without causing any symptoms or apparent injury to the host (Bacon and White 2000). The most frequently encountered endophytes are fungi (Staniek et al. 2009) and basically these organisms are great potential producers of novel, biologically active products (Strobel 2003). These fungal isolates produce a number of bio pharmacological compounds with antimicrobial (Yu et al. 2010), antitumor (Lin et al. 2008) and antiviral properties (Liu et al. 2008). Yet, information allied to their antioxidant activities is very scanty (Strobel 2002).

Fungal endophytes have been recognized as a repository of novel compounds of immense value in agriculture, industry and medicine (Strobel and Daisy 2003). The discovery of novel antimicrobial metabolites from endophytes is an essential alternative to overcome the growing levels

of drug resistance by plant and human pathogens (Yu et al. 2010). Antioxidants act as radical scavengers and inhibit lipid peroxidation and other free radical mediated processes; therefore, these are able to protect the human body from several diseases attributed to the reaction of radicals. The family Rubiaceae includes a well known Genus *Psychotria*, one of the largest genera of flowering plants with estimated 1000–1650 species distributed worldwide (Nepkroeff et al. 1999) has several species of medicinal value. *Psychotria flavida* Talbot. (South Indian Wild Coffee) an endemic shrub, found in all types of forests of Western Ghats is used as medicine for wound healing among tribal people in Southern India (Ayyanar and Ignacimuthu 2009). A number of medicinal properties have been reported in many species of *Psychotria* viz, antibiotic activity in *P. microlabastra* (Khan et al. 2001) and *P. capensis* (McGaw et al. 2000), antiviral activity in *P. serpens* (Kuo et al. 2001) and antiviral/antifungal and anti-inflammatory activities in *P. hawaiiensis* (Locher et al. 1995) and *P. insularum* (Dunstan et al. 2001) respectively.

Very little work has been carried out on endophytes associated with plants of India (Bagchi and Banerjee 2014). The areas of high plant endemicity are expected to possess specific endophytes that may have evolved with endemic

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plant species (Strobel 2002). There is a major need of isolation and bioprospecting of endophytes from medicinally important rare, endemic and threatened plant species of an ethnobotanical history. The present study in this context deals with the enumeration and identification of endophytic fungi associated with *Psychotria flavida* from Western Ghats of Karnataka, India along with primary screening of antimicrobial and antioxidant activities of their extracts. These isolated fungi from the above plant were also studied for biodegradation of plastics and the isolated fungus (*Lasodiplodia theobromae*) possessed potential plastic degrading ability (Sheikh et al. 2015).

Materials and methods

Collection of sample

Healthy, mature, disease free leaf and stem samples of *P. flavida* were collected in 3 different seasons (Summer, Rainy, Winter) from two different locations Charmadi (N 13°04'28.2", E 75°27'39.6" 1823 ft) and Kokkarne (N 13°27'34.6", E 74°50'22.06" 72 ft) during 2012–13. The samples were transferred to sterile bags and brought to the laboratory.

Processing of plant material

The samples were washed thoroughly in running tap water and processed within 24 h of collection. To eliminate epiphytic micro organisms, leaf and stem samples were subjected to surface sterilization (Rosa et al. 2010). The surface sterilized samples were excised into small pieces (0.5 cm size) and plated in potato dextrose agar for more than 10 days. Every season, 100 segments each from both leaf and stem were plated and examined for the endophytic fungal emergence. Few plates were inoculated with surface sterilized sample solution which was used to sterilize the samples in order to check the growth of epiphytes.

Isolation and identification

The emerging fungal tips were sub cultured in potato dextrose agar and identified using molecular and morphological characteristics. One of the isolates from each morphotypes was then selected for molecular identification. DNA was extracted using CTAB method (HiPurA™ Plant DNA isolation kit-HIMEDIA). PCR was conducted to amplify the internal transcribed spacer (ITS) region of the extracted DNA using the primers ITS 1 and ITS 4 (White et al. 1990) under the following conditions: 95 °C for 3 min followed by 35 cycles of 95 °C for 30 s, 50 °C for 45 s, 72 °C for 90 s and final extension at 72 °C for 10 min. PCR amplicons

were electrophorized in 1.2% agarose gel. The amplified PCR products were sequenced by BIOSERVE (Hyderabad) using ABI 3130 (48 capillary) or 3730XI (96 capillary) electrophoresis instrument. BLAST (Basic Local Alignment Search Tools) was used to search for closest match sequences in the GenBank database and the sequences were submitted to gene bank. The non sporulating fungi (mycelia sterilia) were also grouped into morphotypes and identified purely on molecular basis where the fungi were subjected to ribosomal DNA sequence analysis and matched with nucleotide sequences. These similarity values were compared with those of established fungal species and genera obtained from GenBank.

Preparation of extracts for bioactive screening

The fungal isolates were inoculated into Erlenmeyer flasks containing potato dextrose broth and incubated at room temperature under stationary conditions with intermittent shaking for 21 days (Pavithra et al. 2012). To the filtered broth culture and separated mycelia equal volume of ethyl acetate was added, mixed well for 10 min and kept for few minutes till the formation of two clear immiscible layers. By means of separating funnel, the upper layer of ethyl acetate containing the extracted compounds was separated in a separating funnel. The mycelium was grinded properly in a mortar and pestle using ethyl acetate as solvent and then it was filtered using cheese cloth. Filtrate extracts were evaporated to dryness in hot air oven. The extract residue was dissolved in dimethyl sulfoxide (DMSO) and stored at 4 °C to be used as stock solution.

Antioxidant activity

Antioxidant activity was estimated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (Liyana-Pathirana and Shahidi 2005). A solution of DPPH (0.135 mM) in methanol was prepared and 1 ml of this solution was mixed with the fungal extracts of 10 µg ml⁻¹ concentration. The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm using ascorbic acid as standard. The ability to scavenge DPPH radical was calculated as:

% DPPH radical scavenging activity

$$= 100 \times (\text{Absorbance of control} - \text{absorbance of sample}) / (\text{absorbance of control})$$

Antimicrobial activity

Antibacterial and antifungal assay were carried out by the disc diffusion method (Vardar-Unlu et al. 2003). Six

bacterial cultures of which two Gram-positive bacteria *Staphylococcus aureus* (NCIM 2079) and *Bacillus subtilis* (ATCC 6633) and four Gram-negative bacteria *Escherichia coli* (NCIM 2931), *Klebsiella pneumoniae* (NCIM 2957), *Proteus vulgaris* (NCIM 2813) and *Pseudomonas aeruginosa* (NCIM 2200), procured from National Chemical Laboratory, Pune, India were used in the present study. The fungal strain *Candida albicans* MTCC No. 227 obtained from IMTECH, Chandigarh, India were also used.

For in vitro antibacterial activity, 200 µl of overnight grown culture of each bacterium was dispensed into 20 ml sterile nutrient broth and incubated for 4–5 h at 37 °C to standardize the culture to 10⁻⁵ CFU/ml. For about, 0.1 ml (10⁻⁵ CFU/ml) of bacterial culture was inoculated on Muller Hinton agar medium by spread plate technique. Twenty-five microlitre of respective fungal extracts dissolved in 1 ml of DMSO (Dimethyl sulphoxide) were added to the sterile discs (6 mm diameter) purchased from HIMEDIA laboratories individually and aseptically transferred to the inoculated petriplates and incubated for 24 h.

The antifungal activity was assayed by fungal inoculation on to potato dextrose agar (PDA) medium containing discs pre-impregnated with samples. Fungal inoculum was prepared by taking 5–8 colonies of the fresh fungal strain from the petriplate and suspending in 5 ml of sterile distilled water. Hundred microlitre of this fungal inoculum was dispensed into 20 ml of sterile PDA medium, 25 µl of respective samples were added to the sterile discs (6 mm diameter) individually, aseptically and incubated for 2 days in case of *Candida albicans*. Antimicrobial activity was recorded by measuring the diameter of zone of inhibition. Streptomycin and Nystatin (HIMEDIA) were used as positive standards against bacterial and fungal strains respectively.

Analysis of data

Colonization frequency (CF) was calculated as follows:

$$\text{Colonization frequency of endophyte} = \frac{\text{Number of segments colonized by fungi} \times 100}{\text{Total number of segments observed}}$$

The overall colonization rate (CR) was calculated as explained by Petrini et al. (1982):

$$\frac{\text{Total number of leaf segments in a sample yielding} \geq 1 \text{ isolate}}{\text{Total number of leaf segments observed}} \times 100$$

Relative frequencies (RF) of isolation used to represent fungal species density was calculated as the number of isolates of each species of the endophytic fungi divided by the total number of isolates and they were expressed as percentage (Huang et al. 2008). Number of isolates

from tissue segments, divided by the total number of segments was calculated to get isolation rate (IR) (Photita et al. 2001). Shannon and Simpson diversity indices were calculated using online PAST software (Hammer et al. 2001). The frequency of dominant endophytes in each selected plant was calculated as percentage colony frequency divided by sum of percentage of colony frequency of all endophytes $\times 100$ (Kumaresan and Suryanarayanan 2001).

The antimicrobial and antioxidant activities were performed in triplicates (n=3). Statistical analysis was carried out using Graph Pad Prism Software. Statistical differences between extract activities were determined using one way ANOVA with Bonferroni test. Differences were considered statistically significant $p < 0.05$.

Results

Isolation and identification

A total of 1200 segments of leaf and stem samples harboured endophytes throughout the year irrespective of season and locality. In Charmadi, out of 300 each of leaf and stem segments, 147 fungal isolates emerged from leaf segments and 79 isolates from stem segments (Table 1). Similarly, in Kokkarne, out of 300 each of leaf and stem segments 80 isolates emerged from leaf and 74 from stem. The colonization and isolation rates were more in leaf samples compared to stem samples. In the present study, the isolation rates (IR), colonization rates (CR) of endophytic fungi varied with different tissue segments of the medicinal plants studied (Fig. 1).

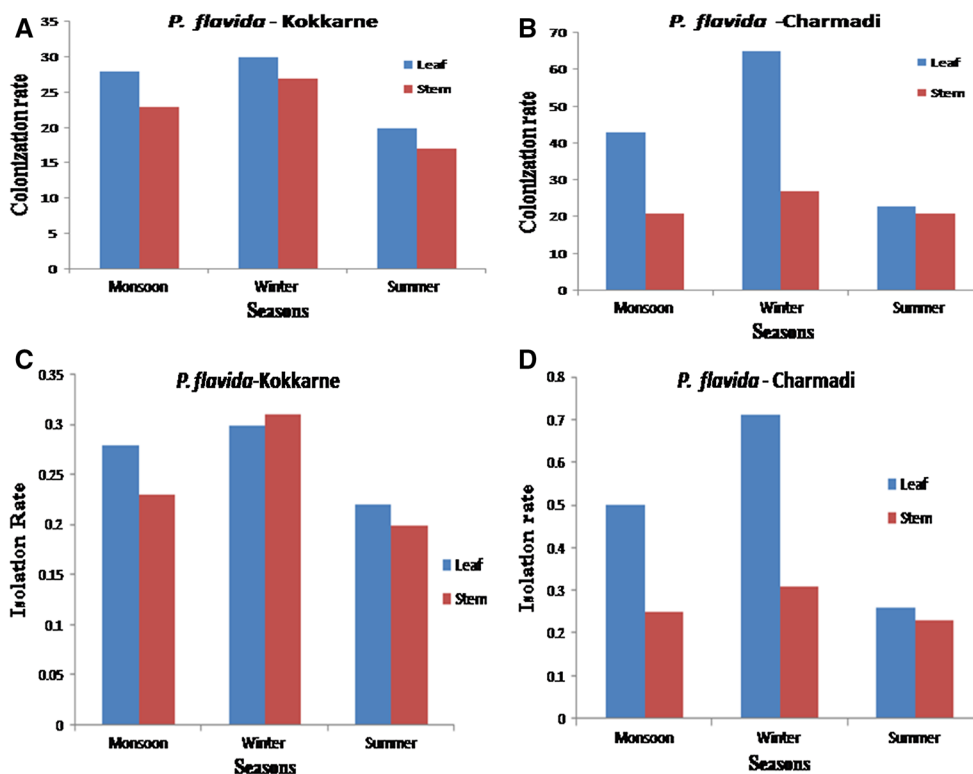
Isolates in the current study included Ascomycetes (10%), Basidiomycetes (10%), Coelomycetes (20%), Eurotiomycetes (10%), Hyphomycetes (20%) and Sordariomycetes (30%). The isolates identified belonged to 10 species. *Talaromyces flavus*, *Cylindrocladium* sp, *Curvularia lunata*, *Phialemonium dimorphosporum*, *Phyllosticta* sp., were found in Kokkarne, *Pestalotiopsis clavispora*, *Phanerochaete* sp., *Bipolaris papendorfii* were exclusively found in Charmadi. *Pichia guillermondii* and *Lasiodiplodia theobromae* were found in both the places. Both foliar and stem endophytic assemblage of *Lasiodiplodia theobromae* was observed with profuse number of isolates prevalently recurring throughout the year in both the places. *Lasiodiplodia theobromae* was dominant among the fungi isolated and the highest frequency of dominance was present in stem segments of *P. flavida* (Table 2). *Pichia guillermondii*, yeast fungi commonly occurred in leaf segments of *P. flavida*. Relative frequency of isolation from the leaf and stem segments of plants is mentioned in Fig. 2. In *P. flavida*, 8 species from leaf and 3 species from stem were isolated. The

Table 1 Percentage of Colonization frequency of endophytic fungi in *P.flavida*

| <i>P.flavida</i> | Colonization frequency % (Number of Isolates) | | | | | | | | | | | |
|--------------------------------|---|---------|---------|---------|---------|---------|----------|---------|---------|---------|---------|---------|
| | CHARMADI | | | | | | KOKKARNE | | | | | |
| | Monsoon | | Winter | | Summer | | Monsoon | | Winter | | Summer | |
| Endophytic fungi | Leaf | Stem | Leaf | Stem | Leaf | Stem | Leaf | Stem | Leaf | Stem | Leaf | Stem |
| <i>L. theobromae</i> | 30 (35) | 21 (25) | 41 (45) | 27 (31) | 20 (23) | 21 (23) | 6 (6) | 13 (13) | 12 (12) | 15 (19) | 16 (18) | 13 (16) |
| <i>P. gullermondii</i> | 3 (3) | – | 3 (3) | – | – | – | – | – | 4 (4) | – | – | – |
| <i>Cylindrocladium sp.</i> | – | – | – | – | – | – | 2 (2) | – | 14 (14) | – | 2 (2) | – |
| <i>P. clavispora</i> | 5 (6) | – | 10 (10) | – | 3 (3) | – | – | – | – | – | – | – |
| <i>C. lunata</i> | – | – | – | – | – | – | 12 (12) | – | – | – | – | – |
| <i>T. flavus</i> | – | – | – | – | – | – | – | 2 (2) | – | – | – | 2 (2) |
| <i>P. dimorphosporum</i> | – | – | – | – | – | – | 8 (8) | – | – | – | 2 (2) | – |
| <i>P. chrysosporium</i> | 4 (5) | – | – | – | – | – | – | – | – | – | – | – |
| <i>B. papendorfii</i> | 1 (1) | – | 11 (13) | – | – | – | – | – | – | – | – | – |
| <i>P. capitallensis</i> | – | – | – | – | – | – | – | 8 (8) | – | 12 (12) | – | 2 (2) |
| Total colonization frequency % | 43 | 21 | 65 | 27 | 23 | 21 | 28 | 23 | 30 | 27 | 20 | 17 |
| Total no of isolates recovered | 50 | 25 | 71 | 31 | 26 | 23 | 28 | 23 | 30 | 31 | 22 | 20 |
| Simpsons index | 0.4816 | 0 | 0.5431 | 0 | 0.2041 | 0 | 0.6837 | 0.552 | 0.6044 | 0.4745 | 0.314 | 0.34 |
| Shannon | 0.9814 | 0 | 1.01 | 0 | 0.3576 | 0 | 1.24 | 0.9022 | 0.9908 | 0.6674 | 0.6002 | 0.639 |
| No. of taxon | 5 | 1 | 4 | 1 | 2 | 1 | 4 | 3 | 3 | 2 | 3 | 3 |

Data based on 100 segments/leaf or stem in each season

Fig. 1 Colonization and Isolation rates of *P. flavida* in Kokkarne (a and c) and Charmadi (b and d)



leaf segments harbored more endophytes than stem segments. *Bipolaris papendorfii*, *Cylindrocladium sp.*, *Phialemonium dimorphosporum*, *Phanerochaete chrysosporium*,

Pestalotiopsis clavispora were found exclusively in *P. flavida* leaf samples. *T. flavus* was found only in stem samples of *P. flavida*.

Table 2 Distribution/dominant frequency of endophytic fungi derived from plant

| Endophytic fungi | <i>P. flavida</i> | |
|--|-------------------|-------|
| | Leaf | Stem |
| <i>Basidiomycetes</i> <i>Phanerochaete chrysosporium</i> | 1.91 | – |
| <i>Hyphomycetes</i> <i>Curvularia lunata</i> | 5.74 | – |
| | 5.74 | – |
| <i>Coelomycetes</i> <i>Lasiodiplodia theobromae</i> | 59.80 | 80.88 |
| | – | 16.16 |
| <i>Ascomycetes</i> <i>Pichia guilliermondii</i> | 4.78 | – |
| <i>Eurotiomycetes</i> <i>Talaromyces flavus</i> | – | 2.94 |
| <i>Sordariomycetes</i> <i>Cylindrocladium sp.</i> | 8.61 | – |
| | 8.61 | – |
| | 4.78 | – |

Antioxidant activity

The crude extracts of fungi and standard ascorbic acid were assessed for their capacity of scavenging free radical using the DPPH radical scavenging assay of concentration 10 µg/ml. After 30 min of incubation, the results (Table 3) showed that 5 of the sample extracts scavenged DPPH radical at 10 µg/ml, while other crude extracts were inactive and showed activity on increase of extract concentration more than 10 µg/ml.

When all the extracts were screened for their antioxidant activity in the DPPH assay, 14 out of 64 extracts (22.2%) were active. In *P. flavida* out of 15 fungi, 5 possessed free

radical scavenging activity at 10 µg/ml, highest being in *L. theobromae* extracts (PFLC, PFLK).

Antimicrobial activity

In the present study, among the 15 crude extracts tested, 11 extracts inhibited the growth of no less than two microbial strains. Zhang et al. (2012) evaluated the antimicrobial activities of extracts from 11 endophytic fungi associated with *Artemisia annua* and concluded that these endophytes could be applied as new sources of antibiotics in agriculture and/or pharmaceutical industries. Huang et al. (2007) reported sixteen endophytic fungal isolates from *Nerium oleander* and tested them for antimicrobial activity. Seven of them exhibited strong antimicrobial activities. *Ascomycetes* sp., *Phoma* and *Colletotrichum* sp. inhibited the growth of at least one test microbe and the isolates of *Torula* sp., *Chaetomium* and *Mycelia sterilia* inhibited the growth of at least two test microbes. None of the extracts showed any activity against *Trichoderma viridae* and *Aspergillus niger*. Antimicrobial activity of ethyl acetate extracts of 15 endophytic fungi isolated from *P. flavida* is given in Table 4. Although the crude fungal extracts exhibited diverse degree of antibacterial activity, the activity was significantly lower as compared to Standard Streptomycin. Conversely, on par higher antibacterial activity of crude extracts of *Lasiodiplodia theobromae* (PFLK) isolated from leaf sample of Charmadi was observed against *K. pneumoniae* but on the other hand, there was zilch anticandidal activity. *Lasiodiplodia theobromae* has been

Fig. 2 Relative frequency of endophytic fungi (a) Leaf (b) stem in *P. flavida*

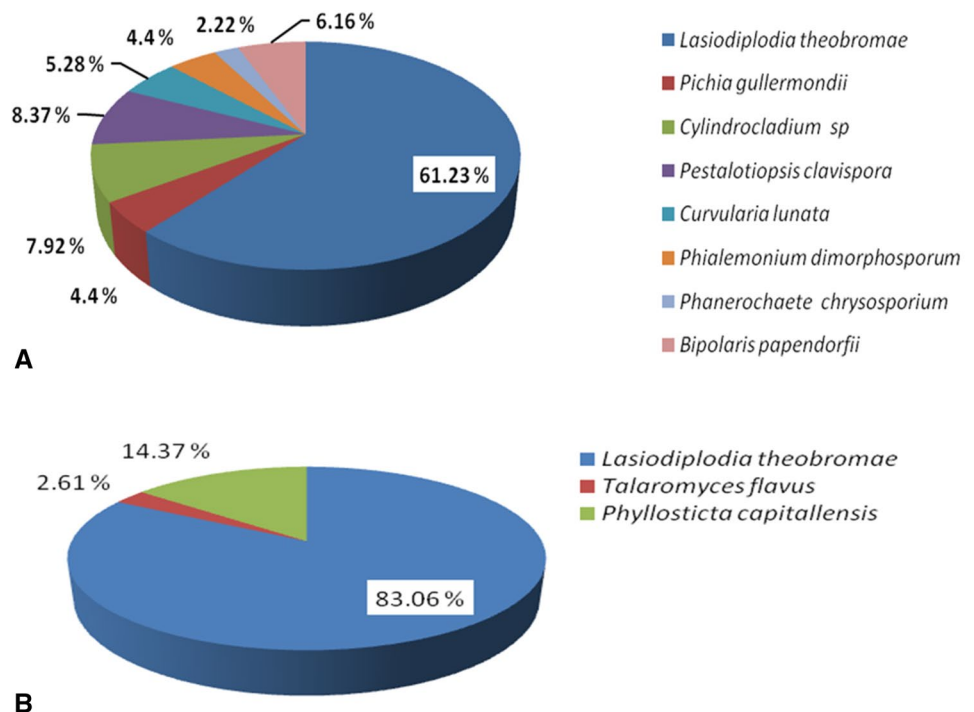


Table 3 DPPH scavenging activity

| ENDOPHYTIC FUNGI | | DPPH (10 µg/ml) % of inhibition |
|----------------------------|------|---------------------------------|
| <i>P. gullermondii</i> | PFLC | – |
| <i>Cylindrocladium sp.</i> | PFLK | 9.79 ± 0.77 ^a |
| <i>P. clavispora</i> | PFLC | 9.75 ± 0.24 ^a |
| <i>C. lunata</i> | PFLK | – |
| <i>T. flavus</i> | PFSK | – |
| <i>P. dimorphosporum</i> | PFLK | – |
| <i>P. chrysosporium</i> | PFLC | – |
| <i>L. theobromae</i> | PFSK | – |
| <i>P. gullermondii</i> | PFLK | – |
| <i>B. papendorffii</i> | PFLC | – |
| <i>L. theobromae</i> | PFSC | 18.39 ± 1.88 ^b |
| <i>P. capitallensis</i> | PFSK | – |
| <i>L. theobromae</i> | PFLC | 50.41 ± 0.15 ^c |
| <i>L. theobromae</i> | PFLK | 49.53 ± 6.11 ^c |
| <i>L. theobromae</i> | PFSK | – |
| Ascorbic acid | | 97.33 ± 0.57 |

PF *P. flavida*, S Stem, L leaf, C Charmadi, K Kokkarne, Values are means of triplicates ($n=3 \pm$ SD). Mean with different superscript in each column are significantly different from each other ($P < 0.05$)

– No DPPH radical scavenging activity at 10 µg/ml

previously reported as endophyte with diverse antibacterial activities in *Piper hispidum* (Orlandelli et al. 2012). Despite the fact that the extracts exhibit broad spectrum of antibacterial activity, only 6 fungal extracts had the potentiality to inhibit the growth of fungi *Candida albicans*. The crude extract of *Phialemonium dimorphosporum* possessed higher antifungal activity (19.66 ± 1.52) but was lower than the standard Nystatin. Qadri et al. (2013) reported from *Artemisia annua* that the extracts from three strains *Fusarium tricinctum* Art, *Gibberella avenacea* Art2 and *Alternaria sp.* Art9, showed significant activity against the fungal pathogen *Candida albicans*. The extract of *Pestalotiopsis clavispora* effectively inhibited all the tested pathogens. Among all the fungal extracts tested from *P. flavida*, *Lasiodiplodia theobromae* exhibited highest antibacterial activity.

Discussion

There was a notably distinct increase in colonization rate and isolation rate of fungi during winter as compared to monsoon and summer. A higher rate of colonization and isolation in the leaf segments was observed compared to stem segments both in monsoon and winter collection in the Charmadi region. However, there was not much difference in the isolation and colonization rates between the leaf and stem during summer.

The isolation rate (IR), colonization rate (CR) and relative frequency (RF) of the endophytic fungi vary with medicinal plants (Huang et al. 2008). Studies carried out previously, have revealed that the species composition and frequency of endophytes vary with different host tissues (Sun et al. 2012). The greater number of total isolates in winter and monsoon seasons than the summer season suggests that colonization by endophytes is associated with climatic factors (Wilson and Carroll 1994). These factors may determine spread and germination success of endophytic fungal spores (Schulthess and Faeth 1998). The composition of the fungal community usually differs between host species (Saikkonen 2007), among the geographically separated individuals of the same host species (Collado et al. 2000) and also within the tissue or organs of a host plant (Kumar and Hyde 2004).

The stem samples of *P. flavida* from Charmadi, recorded the presence of only *L. theobromae* whereas stem samples of Kokkarne recorded the presence of *T. flavus*, *P. capitallensis* and *L. theobromae*. Endophytes can be present as uncommon or single species which are isolated once or very few times, also, by dominant or many species frequently isolated from a given host species (Neubert et al. 2006). During summer season in Charmadi, the colonization frequency of fungi was less probably due to unfavourable factors but it was possible to isolate two species from leaf and one species from stem samples. It may be due to the ability of its spores to survive and even grow at low water potentials. In winter high humidity and moderate temperature may allow the fungal propagules to germinate successfully (Naik et al. 2008). Endophytic fungi are essential components in the biodiversity since they have an effect on structure and defense mechanism of plants and ultimately in the ecosystem (Wilson 2000). In general, colonization rate of leaf samples was found to be higher than stem samples. High colonization rate and richness of species in endophytic fungi was more in leaf segments than stems of host plants sampled from five medicinal species of Kudremukh region of Western Ghats (Raviraja 2005). The colonization (22.66–21.33) and isolation rates (0.12–0.08) of endophytic fungi was recorded from *Vitex negundo* (Sunayana et al. 2014). There was high colonization rates (47.9%–63.1%) and isolation rates (0.7–0.93) observed for endophytic fungi from 1144 tissue fragments of six medicinal plants (Sun et al. 2008). The mean overall colonization and isolation rates of endophytes from *Tripterygium wilfordii* were 57.8 and 65.4% respectively, as stated by Kumar and Hyde (2004). Association of endophytic fungi varies from plant to plant, geographical distribution and also different seasons (Kim et al. 2013). The greater number of total isolates in winter and monsoon seasons than the summer season suggests that colonization by endophytes is associated with climatic factors (Wilson and Carroll 1994). These factors may determine spread and germination success of endophytic fungal spores (Schulthess and Faeth 1998). In

Table 4 Antimicrobial activity of endophytic fungi isolated from *P. flavida* (PF)

| ANTIMICROBIAL ACTIVITY (DISC DIFFUSION METHOD) | | | | | | | | | | | | |
|--|------------------------|-------------------------|--------------------------|-------------------------|--------------------------|-------------------------|-------------------------|-------------------------|------------|------------|---|---|
| ENDOPHYTIC FUNGI | Gram positive Bacteria | | | | Gram negative bacteria | | | | Fungi | | | |
| | B.s | S.a | P.a | K.p | E.c | P.v | C.a | A.n | T.v | | | |
| <i>P. gullermondii</i> | PFLC | - | - | - | - | - | - | - | - | - | - | - |
| <i>Cylindrocycladium</i> sp. | PFLK | 8.66±0.57 ^a | 9.00±0.00 ^a | 8.66±0.57 ^a | 10.33±0.57 ^{ab} | - | 10.66±0.57 ^b | - | - | - | - | - |
| <i>P. clavisporea</i> | PFLC | 11.66±1.52 ^b | 13.33±1.52 ^{bc} | 13.16±1.60 ^d | 11.33±0.57 ^{bc} | 9.00±0.00 ^a | 11.00±1.00 ^a | - | - | - | - | - |
| <i>C. lunata</i> | PFLK | 12.5±0.5 ^b | 13±1.00 ^{bc} | - | 9.66±0.57 ^a | - | - | - | - | - | - | - |
| <i>T. flavus</i> | PFSK | - | - | - | - | - | - | - | - | - | - | - |
| <i>P. dimorphosporum</i> | PFLK | 14.33±0.57 ^c | 12.16±0.28 ^b | - | 12.7±0.60 ^c | 13.00±2.00 ^b | 12.36±0.32 ^a | 19.66±1.52 ^d | - | - | - | - |
| <i>P. chrysosporium</i> | PFLC | - | - | - | - | - | - | - | - | - | - | - |
| <i>L. theobromae</i> | PFSK | 15.16±0.28 ^c | 13.16±0.28 ^{bc} | 10.16±0.2bc | 9.00±0.00 ^a | - | - | 14.66±0.57 ^c | - | - | - | - |
| <i>P. gullermondii</i> | PFLK | - | - | - | - | - | - | - | - | - | - | - |
| <i>B. papendorfii</i> | PFLC | 9.6±0.52 ^a | 12.06±1.79 ^b | - | 11.9±0.17 ^{bc} | - | - | 11.33±0.58 ^b | - | - | - | - |
| <i>L. theobromae</i> | PFSC | 20.66±0.57 ^d | 14.0±0.5 ^c | 11.16±0.28 ^c | - | 8±0.00 ^a | - | - | - | - | - | - |
| <i>P. capitallensis</i> | PFSK | 8.16±0.28 ^a | 8±0.00 ^a | - | - | - | - | - | - | - | - | - |
| <i>L. theobromae</i> | PFLC | 11.16±0.28 ^b | 12.03±0.05 ^b | - | 10.13±0.05 ^{ab} | - | - | - | - | - | - | - |
| <i>L. theobromae</i> | PFLK | 17.66±0.57 ^b | 11.66±0.57 ^b | 13.66±1.15 ^d | 18±0.05 ^c | - | - | - | - | - | - | - |
| <i>L. theobromae</i> | PFSK | 16.00±0.5 ^c | 11.16±0.28 ^b | 9.16±0.28 ^{ab} | 14.66±0.57 ^d | - | - | 8±0.00 ^a | - | - | - | - |
| Streptomycin | | 23.5±0.5 ^e | 22.33±0.57 ^d | 20.66±0.57 ^e | 20.33±0.57 ^f | 18.33±0.57 ^c | 21.33±0.57 ^b | - | - | - | - | - |
| Nystatin | | | | | | | | 24.16±0.76 ^c | 18.16±0.28 | 16.16±0.28 | | |

S Stern, L leaf, C Charmadi, K Kokkame, B.s—*B. subtilis*; S.a—*S. aureus*; P.a—*P. aeruginosa*; K.p—*K. pneumoniae* E.c—*E. coli*, P.v—*P. vulgaris*. Fungi: A.n—*A. niger*, C.a—*C. albicans*, T.v—*T. viridae*

– No. antimicrobial activity. Values are means of triplicates (n = 3 ± SD)

Mean with different superscript are significantly different from each other (P < 0.05)

winter, high humidity and moderate temperature may allow the fungal propagules to germinate successfully (Naik et al. 2008). In the present study, a pronounced increase in the frequency of endophytes was observed during winter compared to monsoon and summer seasons indicating the seasonal variation in the occurrence of endophytes. Foliar endophytes were preferably high in Charmadi than Kokkarne. The endophytes of leaf exhibited greater diversity compared to stem. In the current study, higher Shannon of 1.24 was observed in leaf samples during monsoon in case of Kokkarne followed by 1.01 during winter in case of Charmadi indicating that the high humidity is a reason for diversity Bagchi and Banerjee (2014) reported a highest Shannon–Wiener index (2.494) and Simpson's diversity (0.8926) for *Bauhinia* sp. compared to other three woody lianas. Sunayana et al. (2014) reported in the plant *Vitex negundo* that Simpson's diversity index value ranged between 0.10 and 0.12; the highest value indicated an increase in the species diversity of endophytes. Kim et al. (2013) studied the diversity and seasonal variation of endophytic fungi from three Conifers in Mt. Taehwa, Korea. In the juniper tree, the total H' (Shannon index) was 1.47 and the highest H' (1.00) was observed in April (spring). In the Japanese larch, the H' was 1.74 and the highest H' (1.33) was observed in November (autumn). In the pine tree, H' was 1.58 and the highest H' (1.43) was observed in November (autumn).

In the present study both sporulating and non sporulating fungi were identified based on morphological and molecular identification. Two morphotypes that failed to sporulate even after 3 weeks of incubation showed similarity in their sequences to *Lasiodiplodia* and *Phialemonium*. The sequences of fungi which were identified on molecular level

were submitted to Gene Bank (Table 5). Qadri et al. (2013) isolated a total of 72 strains of endophytic fungi and characterized morphologically as well as on the basis of ITS1-5.8S-ITS2 ribosomal gene sequence acquisition, belonged to 27 representative genera of which, two belonged to Basidiomycota, while the rest of the isolates comprised of Ascomycetous fungi.

DPPH radical scavenging assay at 10 µg/ml of *Lasiodiplodia theobromae* (PFLC, PFLK) was highly active as compared to other extracts but showed lower activity in comparison to Standard Ascorbic acid. Ethyl acetate is often used as an extraction solvent with a considerable selectivity in the extraction of low-molecular-weight phenolic compounds and high molecular-weight polyphenols (Scholz and Rimpler 1989). On the other hand, Conde et al. (2008) have reported that ethyl acetate allowed the highest phenolic content and the selective elimination of non phenolic compounds.

Govindappa et al. (2011) reported the antioxidant activities could be due to strong occurrence of polyphenolic compounds such as flavonoids, tannins, terpenoids phenols and saponins present in endophytic fungi. Moreover, at a concentration of 0.1 mg/ml, that the scavenging activity of ethanol extract of the *A. niger* and *F. oxysporum* reached 88.61 and 86.72% respectively while at the same concentration, *A. flavus* and *F. solani* was 51.66 and 49.13%. Phongpaichit et al. (2007) reported that 22.5% of the extracts of endophytic fungi from *Garcinia* plants exhibited remarkable antioxidant activities.

Endophytic fungi producing natural compounds have been known to inhibit the growth of pathogens (Wiyakrutta et al. 2004). Among the tested fungal organisms in the present study, *Candida albicans* is most sensitive to the extracts

Table 5 Molecular identification of endophytes

| Endophytic fungi whose sequences were submitted to Gene bank | | Accession number | Similarity % (BLAST search) |
|--|------|------------------|-----------------------------|
| <i>Bipolaris papendorfi</i> | PFLC | KF913497 | 99% with KC592365 |
| <i>Curvularia lunata</i> | PFLK | KF913498 | 99% with HF934911 |
| <i>Lasiodiplodia theobromae</i> | PFSK | KF913499 | 98% with KC511597 |
| <i>Lasiodiplodia theobromae</i> | PFSC | KF913500 | 97% with KR183781 |
| <i>Lasiodiplodia theobromae</i> | PFSK | KF913501 | 97% with KT325577 |
| <i>Lasiodiplodia theobromae</i> | PFLC | KF913503 | 93% with JX868680 |
| <i>Lasiodiplodia theobromae</i> | PFLK | KF913505 | 99% with GQ469915 |
| <i>Phyllosticta capitallensis</i> | PFSK | KF913508 | 99% with LC040898 |
| <i>Pestalotiopsis clavispora</i> | PFLC | KF913515 | 99% with KM402033 |
| <i>Phanerochaete chrysosporium</i> | PFLC | KF913520 | 99% with KP771707 |
| <i>Phialemonium dimorphosporum</i> | PFLK | KF913521 | 99% with HM992502 |
| <i>Cylindrocladium</i> sp. | PFLK | KF913525 | 79% with HQ023139 |
| <i>Talaromyces flavus</i> | PFSK | KF913527 | 99% with EF123253 |
| <i>Pichia guilliermondii</i> | PFLK | KF913529 | 99% with KP765045 |
| <i>Pichia guilliermondii</i> | PFLC | KF913530 | 99% with KP764967 |

PF *Psychotria flavida*, L Leaf, S Stem, C Charmadi, A Arboretum, M Herbal garden, K Kokkarne

produced by endophytic fungi. In *P. flavida*, out of 15 representative isolates screened for antifungal activity, only 6 of them showed anticandidal activity. It has been reported that *Pseudomonas aeruginosa* have developed drug resistance towards many antibiotics (Jung et al. 2005). In the present study, however out of 15 fungi, 6 fungi had inhibited *Pseudomonas aeruginosa* in disc diffusion method. Dhankhar et al. (2012) has reported in *Salvadora oleoides* that *Aspergillus* sp. JPY1 showed the inhibition zone of 6.4 ± 0.3 mm against *E. coli*. In the present study, 3 fungi exhibited antibacterial activity against *E. coli*. *Pichia guillermondii*, *Phanerochaete chrysosporium* and *Talaromyces flavus* did not exhibit any antimicrobial activity.

Conclusion

Endophytes hidden within the plant tissue are poorly investigated chemical synthesizers and considered as an important component of biodiversity as the distribution of endophytic mycoflora differs with the host. Endophytic fungi form the promising source for the production of novel products with biological activity. The present study provided promising baseline information for the potential use of the metabolites of endophytic fungi. In addition, the endophytes of these plants may be studied in detail with an ecological perspective which may help to comprehend community structure of their endophytes and warrant isolation of diverse endophytic fungi with useful bioactivities. The present study has opened a vista for addressing the plant and its trapped microorganisms of highly promising future. Promising results obtained from the study warrants further bioprospecting investigations towards the isolation, elucidation and identification of the active principle leading to drug development. With an outlook of safety concerns regarding synthetic compounds, utilization of cheaper and safer source based on natural origin is the focus of contemporary research.

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Compliance with Ethical Standards

Ethical Statement All authors have been personally and actively involved in the study leading to the manuscript, and will hold themselves jointly and individually responsible for its content.

Conflict of interest This manuscript described has not been published before, not under consideration for publication anywhere else and has been approved by all co-authors.

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