MYCOBIOLOGY
2018, VOL. 46, NO. 4, 370–381
https://doi.org/10.1080/12298093.2018.1538068

RESEARCH ARTICLE

OPEN ACCESS Check for updates

Diversity and Bioactive Potential of Culturable Fungal Endophytes of Medicinal Shrub *Berberis aristata* DC.: A First Report

Supriya Sharma, Suruchi Gupta, Manoj K. Dhar and Sanjana Kaul

School of Biotechnology, University of Jammu, Jammu, J&K, India

ABSTRACT

Bioactive natural compounds, isolated from fungal endophytes, play a promising role in the search for novel drugs. They are an inspiring source for researchers due to their enormous structural diversity and complexity. During the present study fungal endophytes were isolated from a well-known medicinal shrub, *Berberis aristata* DC. and were explored for their antagonistic and antioxidant potential. *B. aristata,* an important medicinal shrub with remarkable pharmacological properties, is native to Northern Himalayan region. A total of 131 endophytic fungal isolates belonging to eighteen species and nine genera were obtained from three hundred and thirty surface sterilized segments of different tissues of *B. aristata*. The isolated fungi were classified on the basis of morphological and molecular analysis. Diversity and species richness was found to be higher in leaf tissues as compared to root and stem. Antibacterial activity demonstrated that the crude ethyl acetate extract of 80% isolates exhibited significant results against one or more bacterial pathogens. Ethyl acetate extract of *Alternaria macrospora* was found to have potential antibacterial activity. Significant antioxidant activity was also found in crude ethyl acetate extracts of *Alternaria alternata* and *Aspergillus flavus*. Similarly, antagonistic activity of the fungal endophytes revealed that all antagonists possessed inhibition potential against more than one fungal pathogen. This study is an important step towards tapping endophytic fungal diversity for bioactive metabolites which could be a step forward towards development of novel therapeutic agents.

ARTICLE HISTORY
Received 10 August 2017
Revised 26 December 2017
Accepted 19 February 2018

KEYWORDS
Antagonistic; bioactive; diversity; novel; therapeutic agents

1. Introduction

Endophytes constitute a remarkably multifarious group of microorganisms ubiquitous in plants and maintain an imperceptible association with their host for at least a part of their life cycle. Their enormous biological diversity coupled with their capability to biosynthesize bioactive secondary metabolites has provided the impetus for a number of investigations. Among the microbial group the most frequently isolated endophytes are the fungi. First endophytic fungi was identified and isolated from *Lolium persicum* (Persian darnel) [1]. Endophytic fungi are considered as an outstanding source of bioactive natural products because there are so many of them occupying millions of unique biological niches growing in different types of environment. Plants harbouring fungal endophytes are often healthier than endophyte free ones. Bioactive natural compounds isolated from endophytes have been playing a promising role in the search for novel drugs and have become an inspiring source for researchers due to their enormous structural diversity and complexity [2]. The number of secondary metabolites produced by fungal endophytes is larger than that of any other endophytic microorganism class.

Berberis aristata DC. is one of the most important medicinal shrub native to Northern Himalayan region. It is also known as Indian barberry or tree turmeric. The plant is widely distributed from Himalayas to Srilanka (altitude 6–10,000 ft), Bhutan (altitude 6–10,000 ft) and hilly areas of Nepal in Himalayan regions. It is also found in Nilgiri hills (altitude 6–7000 ft) in South India. It is known locally as Chitra, Daruharidra in Sanskrit, Dar-hald in Hindi, Dar - e - hald in Urdu [3]. *B. aristata* has remarkable pharmacological properties [4].

Almost every part of *B. aristata* has medicinal value for various ailments. *B. aristata* extracts and formulations are useful in the treatment of gynaecological disorders, HIV-AIDS, mitochondrial dysfunction, pharyngitis and various other infections [5,6]. Decoctions of *B. aristata* have also been demonstrated to have antimicrobial activity against a variety of organisms including bacteria, viruses, fungi, protozoans, helminths and Chlamydia [7]. Root and stem of *B. aristata* is used in Indian medical

CONTACT Sanjana Kaul sanrozie@rediffmail.com

preparations, as a hepatoprotector [8,9]. Stem, bark and root extracts have been reported to possess antioxidant, antidiabetic and anti inflammatory activity [10]. Pharmacological studies on the plant show other activities including, anticancer, analgesic and anti-pyretic [11].

Based on the scientific investigations on *B. aristata*, we postulate that these properties may be due to the production of various metabolites by endophytes residing inside the plant. Endophytes have a significant potential as a source of novel bioactive metabolites. Keeping in view the medicinal importance of the host, the present study was carried out to isolate fungal endophytes from various tissues of *B. aristata* and assess their bioactive potential. The present work, to the best of our knowledge is the first report on the isolation of fungal endophytes from *B. aristata* and assessment of their bioactive potential.

2. Materials and methods

2.1. Plant material

The plant material was collected from symptomless plants growing in the fields located at Sial Sui, District Rajouri, J&K, India; Geographical position 33.23 latitude and 74.35 longitude; altitude 2275 feet above sea level. Various plant parts like stem, leaves and roots were collected from healthy and symptomless plants and kept in zip lock bags at 4 °C until further use.

2.2. Isolation of endophytic fungi from the different plant parts of B. aristata

The isolation of endophytes from *B. aristata* was done by using surface sterilization technique [12]. Sterilization involved washing of selected plant parts in running water for 10 min followed by washing twice in distilled water for 1 min and then giving treatment with 70% ethanol for 1 min. Leaves were then treated with 0.5% sodium hypochlorite for 30 s while stem and roots were treated with 0.5% sodium hypochlorite for 1 min. The samples were again treated with 70% ethanol for 1 min. Each step was followed by washing with autoclaved distilled water for 2 min. Surface sterilized plant parts were then dried by pressing them inbetween the folds of sterile filter paper. After proper sterilization, the samples were cut into small segments (0.5–1 cm). The segments were placed on potato dextrose agar plates at 28 ± 2 °C for isolation of the fungal endophytes. Last washing of the surface sterilized segments spread on potato dextrose agar plates served as control. Effectiveness of the surface sterilization method was also validated by Schultz imprinting method [13].

2.3. Identification of endophytic fungi

The endophytic fungi were identified on the basis of morphological and molecular analysis. Molecular identification was done using ITS primers. DNA was isolated using CTAB method [14]. The isolated DNA was air dried, dissolved in TE buffer and stored at −20 °C for further use.

PCR was performed using the primers ITS1 (5′TCCGTAGGTGAACCTGCGG3′) and ITS-4 (5′TCCTCCGCTTATTGATATGC3′). One micro liter of each purified DNA sample (50 ng/μl) was used as a template and added to the PCR master mixture which consisted of 1X PCR buffer, 2 mM $MgCl_2$, 10 mM dNTPs, 1 U/μl Taq polymerase, and 0.1 pM of each primer. The PCR thermal cycle parameters used were 94 °C for 5 min, 30 cycles of 1 min at 94 °C annealing at 55 °C, 1 min at 72 °C, and a final extension at 72 °C for 10 min. PCR reaction was carried out in Mastercycler gradient machine (Eppendorf, Hamburg, Germany). The amplified products were checked on 2% agarose gel, eluted and sequenced. The obtained sequences were compared with NCBI databases using the BLAST algorithm. Sequences were submitted to GenBank and accession numbers were obtained. Phylogenetic analysis were conducted using MEGA version 6.

2.4. Fermentation and extraction of secondary metabolites from fungal endophytes

Submerged fermentation of the endophytic fungi was done for the production of secondary metabolites [15]. Four agar plugs (8 mm diameter) of actively growing fungal culture were inoculated into 200 ml of autoclaved potato dextrose broth in 1000 ml of Erlenmeyer flasks. The flasks were then incubated at 28 °C under continuous shaking for 14 days at 120 rpm. Flasks were taken out on the 15th day from the incubator shaker. The fungal biomass was filtered using autoclaved glass funnel, filter paper and muslin cloth. Fungal metabolites were extracted by solvent fractionation and Soxhlet extraction methods using ethyl acetate and 80% ethanol respectively.

2.5. Antibacterial activity

Crude ethyl acetate and ethanolic extracts of the fungal isolates were evaluated for their antibacterial potential against some common human pathogenic microorganisms. The bacterial cultures were obtained from MTCC: *Pseudomonas aeruginosa* (MTCC-1934), *Bacillus subtilis* (MTCC-441), *Klebsiella pneumonia* (MTCC-109), *Pseudomonas alkaligenes* (MTCC-493), *Staphylococcus aureus, Enterococcus faecalis* (MTCC-439), *Bacillus cereus*

(MTCC-430), and *Escherichia coli* (MTCC-40). Antibacterial potential of the extracts was assessed using well diffusion method [16]. Specific number of wells were punched with a sterile cork borer (6 mm diameter) in each plate. 100 µl of various concentrations of the test samples and control (positive and negative) were added seperately into wells. Dimethyl sulphoxide (DMSO) was used as a negative control and standard antibiotic chloramphenicol (10 µg/ml) was used as the positive control. The plates were allowed to stand for 1 h for diffusion of the extracts followed by incubation for 24 h at 37 ± 2 °C. The antagonistic potential of different extracts of endophytic isolates was determined in terms of diameter of clear bactericidal zone around the well. The crude extracts which showed clear zone around the wells were considered as positive whereas those not showing clear zone around the well were considered as negative. Minimal inhibitory concentration (MIC) of the selected crude extracts of the fungal endophytes showing more than 10 mm of the clear zone was determined using concentrations ranging from 50 to 1000 µg/ml, against specific pathogenic bacteria.

2.6. Antifungal activity

Antagonistic potential of the endophytic fungal isolates was assessed against common fungal pathogens obtained from MTCC: *Microsporum canis* (MTCC-3270), *Candida albicans* (MTCC-7253), *Alternaria alternata* (MTCC-2724), *Penicillium chrysogenum* (MTCC-6891), *Aspergillus fumigatus* (MTCC-3926), *Colletotrichum capsici* (MTCC-3414), and *Candida glabrata* (MTCC-3019) using the dual culture method [17] with slight modifications. In dual culture technique both the test pathogen and the anticipatory antagonist were made to grow on a same plate separated by some distance. The antagonistic potential of the latter was determined in terms of reduction in the radial growth of test pathogen in presence of antagonist. Actively growing cultures of endophytic fungal isolates, were inoculated on one side of a PDA plate. The culture was allowed to grow for three days and the culture agar plug from actively growing pathogen culture was placed at a distance of 6 cm from the preplaced growing antagonist. Agar plugs of endophytic fungal isolates, inoculated on one side of a PDA plate served as control. The plates were then incubated at 28 ± 2° C. After 10 d, the growth diameter of pathogenic culture in presence of antagonist as well as in absence of antagonist was measured. The percentage growth inhibition (PGI) of the fungal pathogens by different antagonists (fungal endophytes) was determined by using the formula [18]

$$\mathrm{PGI} = [(R_1 - R_2)/R_1] \times 100$$

where "R_1" is the radial growth of fungal pathogen in the absence of antagonist; "R_2" is the radial growth of fungal pathogen in the presence of antagonist. The PGI was categorized on growth inhibition category (GIC) scale from 0 to 4 where 0 = no growth inhibition; 1 = 1–25% growth inhibition; 2 = 26–50% growth inhibition; 3 = 51–75% growth inhibition; 4 = 76–100% growth inhibition. Based on PGI endophytes were considered as antagonists. Antagonist showing PGI value greater than 50% were considered as potential biocontrol agents against the fungal test pathogens.

2.7. Antioxidant activity

The crude extracts of the endophytic fungal isolates were screened for antioxidant activity using DPPH radical scavenging assay [19]. Different concentrations of the extracts dissolved in methanol (0.5 ml) were mixed with 1 ml of a methanolic solution of 0.2 mM DPPH (Sigma Aldrich, St. Louis, MO). After mixing thoroughly, the mixture was allowed to stand in the dark for 30 min and the absorbance was measured at 523 nm using methanol for the baseline correction. The results were then compared with those of the control prepared as above but without any sample. Radical scavenging activity was expressed as a percentage and was calculated using the following formula:

$$\%\ \text{Scavenging} = [(\mathrm{A_{control}} - \mathrm{A_{sample}})/\mathrm{A_{control}}]\ 100$$

where A: Absorbance

2.8. Statistical analysis

The colonization frequency of endophytic fungi was calculated using the formula:

$$\mathrm{CF}(\%) = (N/N\mathrm{t})100$$

where *N*: Number of isolates obtained; *N*t: Total number of segments inoculated.

Similarity indices were used to compare similarity of endophytic fungal assemblage among various tissues. Sorenson's index of similarity, QS = 2a/(2a + b + c + d), where a is the number of common species of endophytic fungi in leaf, stem, and root tissues and b, c, d correspond to the species specified to leaf, stem and root tissues respectively. Jaccard's index of similarity was calculated using the formula, JS = a/(a + b + c + d), where "a" is the number of fungal endophytes common in leaf, stem and root tissues whereas b, c, and d correspond to the species specific to leaf, stem and root tissues respectively.

The fungal diversity among mycological populations was estimated using various diversity indices. Simpson's index of dominance (D) was calculated using the formula, $D = \sum (n/N)^2$, where *n*: Total number of isolates of particular species: *N*: Total number of isolates of all species. Simpson's diversiy index = 1-D. Species richness = $S/(N)^{1/2}$, where S: Total number of species. The Shannon–Wiener index (H') was calculated as ($p_i \ln p_i$), where p_i: *n*/*N*. Species evenness (E) was evaluated by H'/lnS. The range of antibacterial activity of endophytic isolates was calculated as the number of bacteria inhibited by each fungal endophyte divided by the total number of bacteria tested and multiplied by 100.

3. Results and discussion

3.1. Isolation and identification of fungal endophytes

Endophytes have been found virtually in every plant studied, where they colonize the internal tissues of their host plant and can form a range of relationships including symbiotic, mutualistic and commensalistic. Endophytes residing within medicinal plants are exploited more as compared to other plants as they are considered as the rich source of bioactivemetabolites [20]. A few reports are available on exploration of fungal endophytes, which may be due to their slow growth rate and strenuous handling [21]. Biological activities of the plants can be attributed to the endophytes inhabiting within them [22]. Literature survey has shown that many medicinal plants have been explored for their fungal endophytes. However, to the best of our knowledge there is no report available for *B. aristata*. Therefore, the present study is the first report on the isolation and identification of fungal endophytes from *B. aristata*.

A total of 131 endophytic fungal isolates belonging to 18 species and 10 genera were obtained from 330 surface sterilized segments of different tissues of *B. aristata*. In terms of total isolates under endophytic taxa, 10 were isolated from leaves, three from

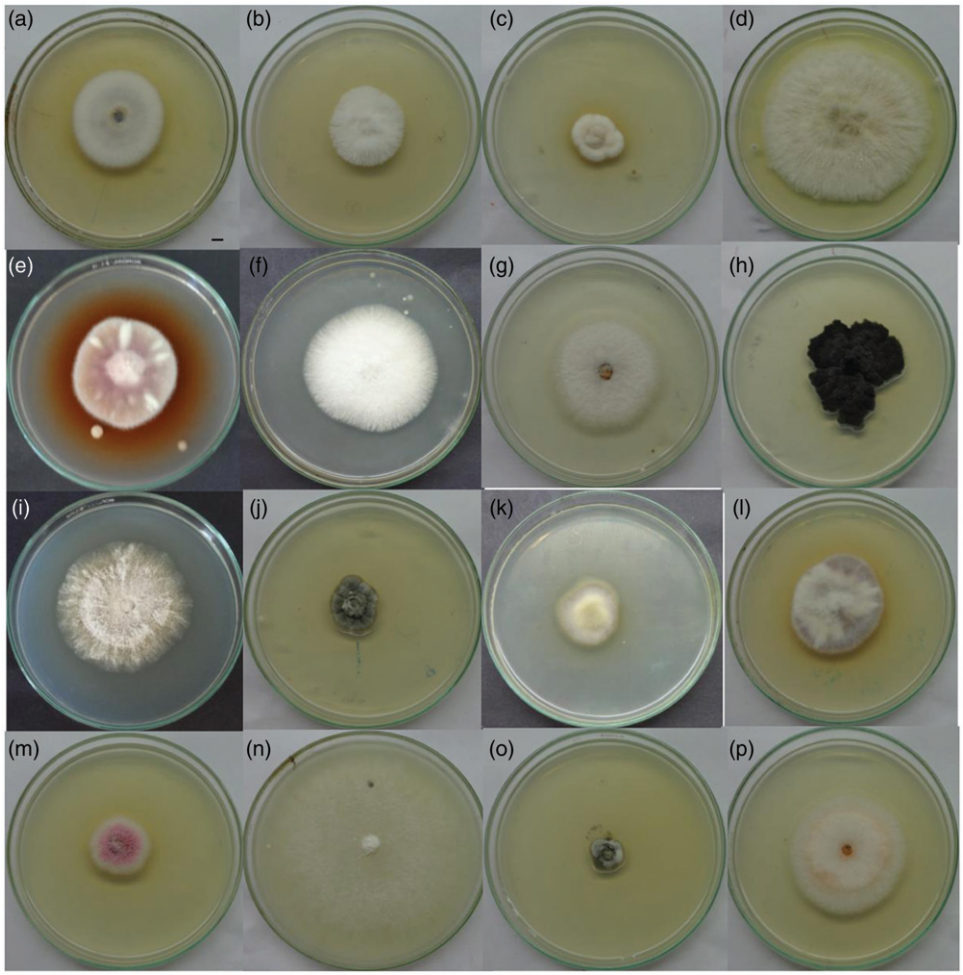

Figure 1. Colony characteristics of eight days old endophytic fungal isolates of *Berberis aristata* on potato dextrose agar.(a) *Fusarium solani* (A1), (b) *Colletotrichum coccoides* (A2), (c) *Fusarium nematophilum* (A3), (d) *Clonostachys rosea* (A4), (e) *Fusarium solani* (A5), (f) *Colletotrichum gleosporioides* (A6), (g) *Colletotrichum kahawae* (A7), (h) *Phyllosticta capitalensis* (A8), i) *Phomopsis* spp. (A9), (j) *Aspergillus flavus* (A10), (k) *Alternaria macrospora* (S2), (l) *Alternaria solani* (S3), (m) *Fusarium lateritium* (S6), (n) *Cercospora citrullina* (S7), (o) *Colletotrichum gleosporioides* (S8), (p) *Myrothecium inundatum* (S9).

Table 1. Colonization frequency of the endophytic fungal isolates of *Berberis aristata* DC.

Accession No.	Fungal isolates	Plant tissue	Total number of segments incubated	No. of isolates obtained	Colonisation frequency	GenBank accession no.
S1	*Alternaria macrospora*	Leaf	110	15	13.6	KXO99951
S2	*Alternaria solani*	Leaf	110	11	10	KXO99952
S3	*Fusarium falciforme*	Root	110	3	2.7	KX138450
S4	*Fusarium oxysporum*	Stem	110	1	0.9	KX138451
S5	*Fusarium solani*	Root	110	4	3.6	KX138452
S6	*Fusarium lateritium*	Stem	110	10	9	KX138453
S7	*Cercospora citrullina*	Stem	110	7	6.3	KX138454
S8	*Colletotrichum gleosporioides*	Leaf	110	4	3.6	KX138455
S9	*Myrothecium inundatum*	Leaf	110	8	7.2	KX138456
A1	*Fusarium solani*	Root	110	15	13.6	KX138457
A2	*Colletotrichum coccoides*	Root	110	5	4.5	KX138458
A3	*Fusarium nematophilum*	Root	110	12	10.9	KX138459
A4	*Clonostachys rosea*	Root	110	3	2.7	KX138460
A5	*Fusarium solani*	Root	110	6	5.4	KX138461
A6	*Colletotrichum coffeanum*	Leaf	110	5	4.5	KX138462
A7	*Colletotrichum kahawae*	Leaf	110	7	6.3	KX138463
A8	*Phyllosticta capitalensis*	Leaf	110	2	1.8	KX138464
A9	*Phomopsis tersa*	Leaf	110	4	3.6	KX138465
A10	*Aspergillus flavus*	Leaf	110	2	1.8	*KY569252*
A11	*Alternaria alternate*	Leaf	110	7	6.3	KX138466

stem and seven from root segments. Leaf segments were found to inhabit more endophytes than segments of other tissues (Figure 1). These fungal endophytes were identified on the molecular basis using internal transcribed spacer regions and 5.8S rRNA gene sequencing and NCBI-BLAST algorithm. BLAST search yielded high homology with the respective species in the GenBank database from NCBI. Molecular identification of the selected fungal endophytes on the basis of sequence analysis revealed that endophytic fungi belonged to 10 genera and 18 different species (Table 1).

In leaf tissues *Alternaria macrospora* was found to have highest colonization rate (13.6%), in roots *Fusarium solani* with highest colonization rate (13.6%) whereas *F. lateritium* from stem was ranked first in terms of its colonization rate (9%). Species of *Alternaria* and *Aspergillus* were restricted only to leaf tissues, whereas *Fusarium* spp. were restricted to stem and root tissues. One of the possible reasons for the difference in the colonization rate between plant parts is the structure and substrate which influences the colonization and distribution of endophytic fungi [23].

During the present study *Fusarium* spp. was the dominant taxa. It preferentially colonized the host stem and root tissues. Previous studies have also indicated the host-species specificity of the endophytes [24]. However, there are other factors like soil conditions, climate and the dynamics of soil microflora that may also influence the colonization of endophytes in the plant tissues [23,25]. The most frequently isolated fungal endophyte was *F. solani*. It has also been reported as an endophyte in many other medicinal plants [26,27]. Although, a number of fungal species can be recovered from the plant, only a few are found frequently and become dominating genera of the host species [28].

In the current study *A. macrospora* was isolated as an endophyte from the leaves of *B. aristata*. Previously, it has been isolated as a pathogen that infects mostly *Brassica* spp. including some important crops such as broccoli, cabbage, and oil seed rape [29]. There are no reports available on its isolation as an endophyte from any medicinal plants. To the best of our knowledge, there is no report on isolation of *A. macrospora* as an endophyte. The difference in endophytic assemblage in different tissue types might be a capacity for utilizing the substrate along with factors like tissue physiology and chemistry [24]. The larger surface area of the leaves and the presence of stomata may provide passage to the entry of fungal mycelia, harboring different endophytic fungi [30,31]. Many species of *Alternaria* are common epiphytes, but can also occur as endophytes as supported by the work of many workers [32]. Some of the isolates obtained in the current study like *Myrothecium inundatum*, *A. alternata, A. solani, F. oxysporum, F. solani, Phomopsis tersa, Colletotrichum coccoides, C. gleosporioides, Phyllosticta capitalensis, Clonostachys rosea,* and *Aspergillus flavus* are common fungal endophytes. Earlier reports have also mentioned the isolation of these endophytes from other medicinal plants [33–35].

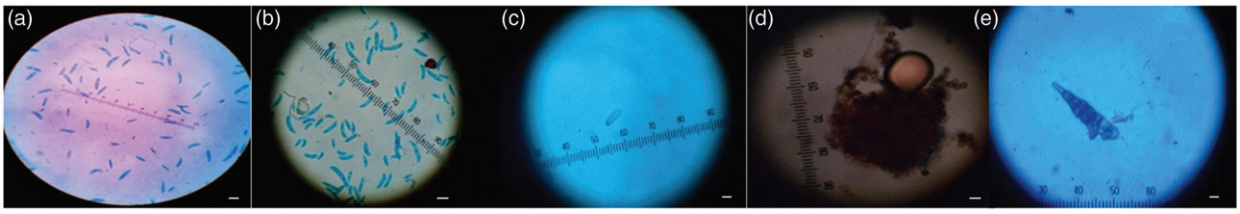

Figure 2. Conidial characteristics of some of the fungal endophytes of *Berberis aristata*. **(a)** *Fusarium solani* (A1), (b) *Fusarium solani* (A5), (c) *Colletotrichum kahawae* (A7), (d) *Aspergillus flavus* (A10), (e) *Alternaria alternata* (A11).

The phylogenetic tree was constructed using Neighbourhood joining method (Figure 2). It depicted evolutionary relationship among taxa of endophytic fungal isolates with the reference taxa obtained from GenBank. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) were shown above the branches and the clades formed were supported by bootstrap value >50%. All of the 20 fungal endophytes in the phylogenetic tree belonged to the phylum Ascomycota, which is in consensus with the statistical results showing that Basidiomycota endophytes were seldom found within plants [36]. These isolates were further divided into eight groups on the basis of genera attained by the fungal isolates and these eight groups belonged to three different classes namely Sordariomycetes, Dothiomycetes, and Eurotiomycetes. The isolates A1, A5, and S5 shared a clade with *F. solani* while the isolate S3 with *F. falciforme* showing 100% similarity. Similarly, the isolates S6, S4, and A3 were grouped with *F. lateritium, F. oxysporum,* and *F. nematophylum* respectively with bootstrap value of 100%. The isolates A1, A5, S5, S3, S6, S4, and A3 belonged to the group I. The isolates A2 and A4 belonged to group II, and showed similarity with *C. coccoides* and *C. rosea*, respectively. The isolates A6 and A7 formed a common clade with *C. gleospoiroides* and S8 with *C. kahawae* of group IV showing 58–100% similarity. A9 and S9 of group III and VII showed 100% bootstrap value with *P. tersa* and *M. inundatum*, respectively. The isolates of group I, II, III, IV, and VII belonged to the class Sordariomyces.

The group VI isolates S1, S2, and A11 shared a clade with *A. macrospora, A. solani,* and *A. alternate*, respectively with bootstrap value ranging from 90–100%. Similarly, the isolates S7 and A8 formed a clade with *Cercospora citrullina* and *Phylosticta capitalensis* respectively belonging to the group V. These isolates belonged to the class Dothideomycetes. The isolate A10 of group VIII formed a clade with *A. flavus* and belonged to a separate class Eurotiomycetes. The phylogenetic analysis therefore, depicted diverse taxonomic affinities among the isolated fungal endophytes of *B. aristata.*

The diversity of endophytic community isolated from various tissues was compared using indices of

Table 2. Diversity indices of endophytic fungi colonizing leaf, root and stem tissues of *Berberis aristata* DC.

Indices	Leaf	Stem	Roots
Simpson's dominance	0.149	0.457	0.192
Simpson's diversity	0.851	0.549	0.807
Species richness	1.240	0.700	0.722
Shannon–Weiner	2.130	0.839	1.517
Evenness	0.920	0.770	0.940

α-diversity: Shannon–Wiener index and Simpson's diversity index and their components like species richness and evenness. Simpson's dominance of endophytic fungi was higher in stem tissues. Similarly, Simpson's diversity and Shannon-–Wiener indices were higher in fungal endophytes of leaf tissues. There was a slight difference between the species evenness of the fungal endophytes of leaf and root tissues (Table 2). Since, there was no similarity among the fungal endophytes of various tissues of the *B. aristata*, the values of Jaccard's and Sorenson's similarity indices for endophytic fungi of different tissues were found to be zero.

3.2. Antibacterial activity

Different degree of antibacterial activity was recorded in crude ethyl acetate and ethanolic extracts of the twenty endophytic fungal isolates at concentration of 1 mg/ml against both Gram positive and Gram negative bacteria (Table 3). The extracts showing potent antibacterial activity (diameter of clear zone above 10 mm) were further selected for the determination of MIC. MIC of the ethyl acetate extract in case of Gram negative bacteria ranged between 0.05–1mg/ml and Gram positive bacteria ranged between 0.05–0.8 mg/ml. The results revealed that ethyl acetate extracts were more effective than ethanolic extracts. The possible reason for this might be the superior extraction values of ethyl acetate extracts as compared to ethanolic extracts. Ethyl acetate extracts of the fungal endophytes exhibited broad spectrum activity as indicated by their bacterial inhibition potential (Figure 2). The inhibition of the pathogens by crude extracts might be due to the production of biologically active compounds in media [37].

The ethyl acetate extracts of *A. macrospora* and *F. solani* were effective against all test pathogens

Table 3. Minimum inhibitory concentration (MIC) of selected endophytic fungal extracts against specific pathogenic bacteria.

			Diameter of clear zone (in mm)								
			Conc. of extracts (in µg/ml)								
Fungal isolates	Bacterial pathogens	Extract	1000	800	500	300	200	100	50	+ve C	−ve C
Alternaria macrospora	*Pseudomonas alcaligenes*	EA	21	19.5	18.5	18	17	16	15	26	−
Alternaria macrospora	*Pseudomonas aeruginosa*	EA	18.5	17.5	17	16.5	15	14.5	13	19	−
Alternaria macrospora	*Bacillus subtilis*	EA	11	6.5	−	−	−	−	−	23	−
Alternaria macrospora	*Bacillus cereus*	EA	11	10.5	10	9.5	9	8.5	8	18	−
Alternaria macrospora	*Klebsiella pneumonia*	EA	20	19.5	18	17.5	17	16	15	24	−
Alternaria macrospora	*Enterococcus faecalis*	EA	12.5	11.5	10	9.5	8.5	8	+	19	
Clonostachys rosea	*Bacillus subtilis*	E	21	19.5	19	18.5	17.5	16.5	14	23	−
Clonostachys rosea	*Pseudomonas aeruginosa*	E	10.5	9	8.5	8	+	+	+	19	−
Myrothecium inundatum	*Pseudomonas alcaligenes*	EA	10	8.5	8	−	−	−	−	26	−
Fusarium solani	*Bacillus subtilis*	EA	9.5	9	8.5	7	+	+	+	23	−
Fusarium solani	*Escherichia coli*	EA	13.5	13	11	+	−	−	−	15	
Fusarium solani	*Staphylococcus aureus*	EA	12	11	10	9.5	9	−	−	20	−
Colletotrichum coffeanum	*Bacillus cereus*	EA	10	9.5	9	8.5	8	7	6.5	18	−
Colletotrichum kahawae	*Escherichia coli*	EA	9	+	−	−	−	−	−	15	−
Aspergillus flavus	*Klebsiella pneumonia*	E	10.5	10	9.5	9	8.5	+	+	24	−

+ve C: Chloramphenicol; −ve C: DMSO; E: Ethanol; EA: Ethyl acetate.

except *S. aureus* and *B. cereus*, respectively (Figure 3). The activity of ethyl acetate extract of *A. macrospora* against *P. aeruginosa* was almost as effective as chloramphenicol and the activity of ethyl acetate extract of *F. solani* against *P. aeruginosa* was in close proximation with chloramphenicol. Antimicrobial activity of various *Alternaria* spp. has been reported [38] but to the best of our knowledge this study forms the first report on antimicrobial activity of *A. macrospora*.

In the present study ethyl acetate extracts of *F. solani* were found to be more effective against Gram positive bacteria. The resistance of the Gram-negative bacteria could be attributed to its cell wall structure. It has been found that Gram-negative bacteria have an effective permeability barrier, comprised of a thin lipopolysaccharide exterior membrane, which could restrict the penetration of the crude extracts whereas, Gram-positive bacteria have a mesh-like peptidoglycan layer which is more accessible to permeation by the crude extracts [39,40]. Similarly, antimicrobial activity of *F. solani* against Gram-positive bacteria has been reported [41]. Highest inhibitory activity of endophytic *Fusarium* spp. against Gram-positive bacteria (*B. subtilis* and *S. aureus*) than Gram-negative bacteria (*E.coli*) has been reported [42]. In contrast to our study, the previous studies have reported that *F. solani* does not show antibacterial activity against Gram-positive and Gram-negative bacteria [43].

The activity of ethanolic extract of *C. rosea* against *B. subtilis* was in close proximation with the activity of chloramphenicol. Similarly, antimicrobial activity of *C. rosea* against Gram -positive bacteria has been reported [44]. *C. rosea* is known to produce a wide range of secondary metabolites that possess strong antibacterial potential [45]. Recently, three new bisorbicillinoids have been isolated from *C. rosea* YRS-06 that exhibit strong antibacterial activity against pathogenic bacteria [46].

3.3. Antifungal activity

Antifungal potential of 20 fungal endophytes against more than one pathogens was reported (Table 4). *In vitro* dual culture method was followed to assess the antagonistic potential of the fungal endophytes. The most promising activity was observed in *F. solani* and *F. falciforme* against *A. fumigatus* with highest PGI value (60%). Similar results were observed wherein antifungal activity of *F. solani* and other *Fusarium* spp. against *A. fumigatus* using well diffusion technique was determined [47]. Similarly, antifungal activity of *F. solani* against *A. alternata* and *Penicillium* spp. has been reported [48]. There are various reports in literature where antagonistic potential of the fungal endophytes has been reported against *Fusarium* spp. but only a few reports are available where *Fusarium* spp. have been used as an antagonist against common fungal pathogens viz, *A. fumigatus, M. canis, P. chrysogenum, C. albicans* using dual culture technique.

Twelve antagonists exhibited inhibition potential against almost all the test pathogens, thus belonging to different GICs. Most succeptible pathogens were *A. fumigatus* and *C. kahawae* whose growth was inhibited upto 51% by *C. kahawae*, 52% by *P. tersa* and *C. gleosporioides*, 60% by *F. solani* and *F. falciforme,* and 53% by *C. kahawae* respectively. *C. coffeanum, C. kahawae* and *C. gleosporioides* were earlier reported only as pathogens of coffee as it cause anthracnose, the main limiting factor of coffee. However, during the present study we found the antagonistic potential of endophytic *C. coffeanum* and *C. kahawae* against fungal pathogens. Antagonistic potential of *Colletotrichum* spp. have

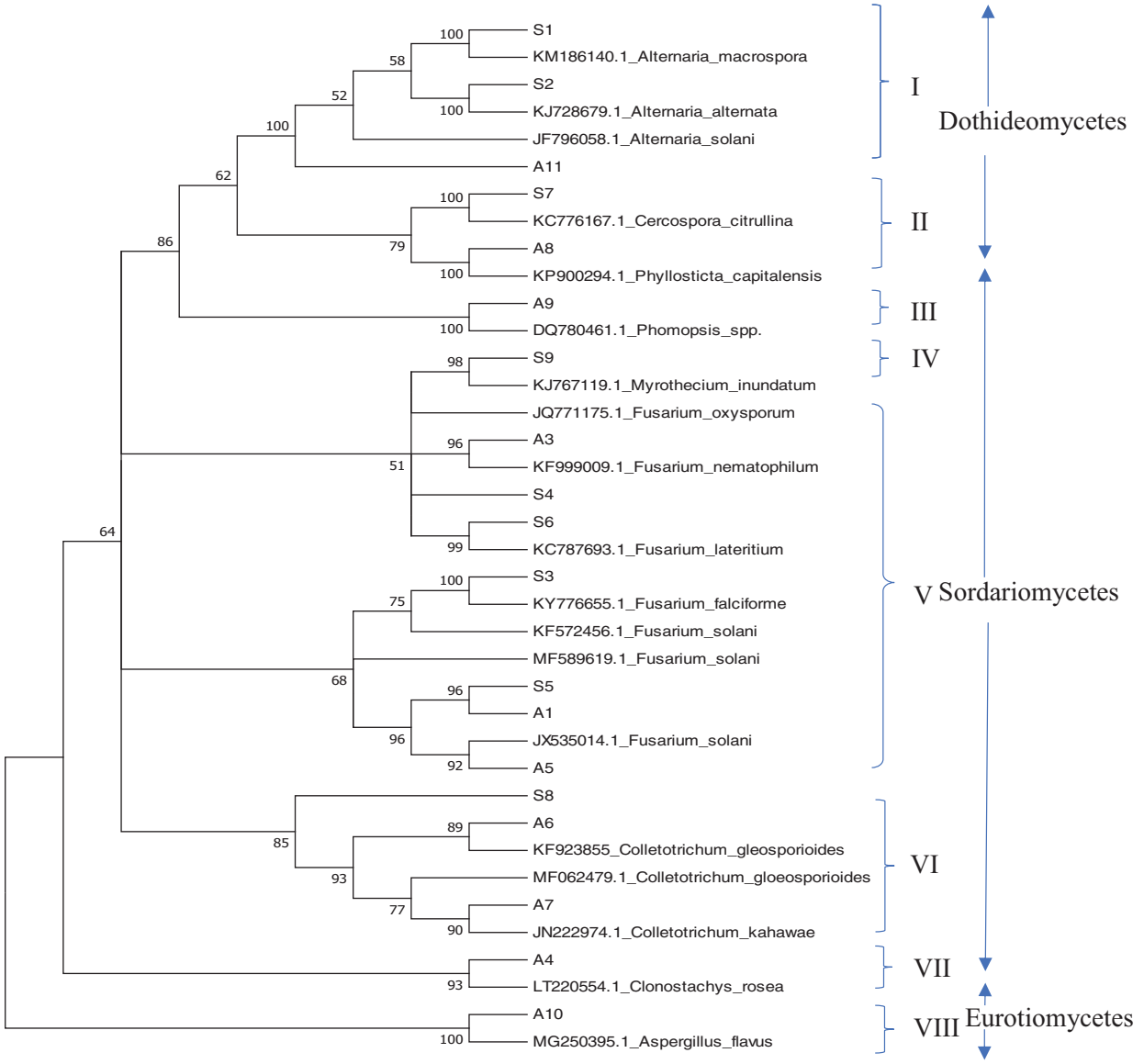

Figure 3. Evolutionary relationship among taxa of endophytic fungal isolates obtained from *Berberis aristata* with the reference taxa.

also been reported against various fungal pathogens [49,50].

3.4. Antioxidant activity

The crude extracts exhibited a concentration dependent increase in free radical scavenging activity. Even though the DPPH scavenging aptitude of the extracts was found to be lower than that of the commercial antioxidant ascorbic acid, it still reached at a percentage inhibition above 80% at 1 mg/ml concentration in some of the crude extracts. The crude ethyl acetate extract of *A. flavus* exhibited 81.4% DPPH radical scavenging activity at a concentration of 100 µg/ml with IC_{50} value of 39 µg/ml as compared to 17.66 µg/ml, the IC_{50} value of ascorbic acid, followed by, ethyl acetate extract of *A. flavus* and *A. alternata* which exhibited IC_{50} value of 40 µg/ml suggesting the potentiality of endophytic fungal extracts as antioxidants (Table 5).

Oxidative stress produced in the body due to various factors has been known to have a significant effect in the causation of Alzheimer's disease, cancer, cardiovascular disorders, diabetes ,and diabetes related complications in human beings. In our study promising antioxidant potential was observed in crude extracts of *A. flavus* and *A. alternata* showing that these endophytic extracts have the proton donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants. The antioxidant properties of their crude extracts may be attributed to the presence of total phenolics and flavonoids therein [50]. Different compounds with antioxidant potential have been isolated from fungal endophytes [51]. The observed lower IC_{50} values of these extracts support the significance of fungal endophytic

Table 4. Percentage growth inhibition of the pathogenic fungal cultures by endophytic fungal isolates of *Berberis aristata*.

	Fungal isolates																		
Fungal pathogens	*Fusarium solani*	*Colletotrichum coccoides*	*Fusarium nematophilum*	*Clonostachys rosea*	*Fusarium solani*	*Colletotrichum coffeanum*	*Colletotrichum kahawae*	*Phyllosticta capitalensis*	*Phomopsis tersa*	*Aspergillus flavus*	*Alternaria alternata*	*Alternaria macrospora*	*Alternaria solani*	*Fusarium falciforme*	*Fusarium oxyspoum*	*Fusarium solani*	*Fusarium lateritium*	*Cercospora citrullina*	*Colletotrichum gleosporioides*	*Myrothecium inundatum*
	% growth inhibition of pathogenic fungi																		
Microsporum canis	37	47	23	33	29	38	18	40	33	22	12	13	27	34	19	26	42	40	33	28
Alternaria alternata	50	40	23	29	29	46	37	41	37	44	21	19	46	37	48	12	50	12	16	14
Candida albicans	35	35	29	23	23	23	18	23	19	22	–	–	–	–	–	–	–	26	35	–
Penicillium chrysogenum	29	12	18	18	18	47	35	20	29	41	23	12	32	23	35	26	12	35	29	18
Colletotrichum capsicii	40	30	3	39	44	50	53	28	42	33	29	14	22	44	8	11	36	28	58	31
Aspergillus fumigatus	60	46	33	48	57	52	51	44	52	48	36	43	36	60	34	27	38	25	52	44

Table 5. DPPH radical scavenging activity of the selected endophytic fungal extracts of *Berberis aristata* and their IC_{50} value.

		Fungal isolates											
	Control	*Fusarium solani*	*Colletotrichum coccoides*	*Fusarium nematophilum*	*Clonostachys rosea*	*Colletotrichum coffeanum*	*Colletotrichum kahawae*	*Phyllosticta capitalensis*	*Phomopsis tersa*	*Alternaria alternata*	*Alternaria alternate*	*Fusarium falciforme*	*Fusarium lateritium*
Extract	–	EA	EA	EA	EA	EA	EA	EA	EA	EA	E	EA	EA
Conc. (μg/ml)	50	1000	1000	1000	1000	1000	100	1000	100	100	1000	1000	1000
%DPPH radical scavenging	93.4	82	88	83	86.5	85	60	83	81.4	85.2	82	34	410
IC_{50} (μg/ml)	17.6	472	245	374	180	426	87	406	87	40	442	78	457

EA: Ethyl acetate; E: Ethanol; IC_{50}: Half maximal inhibitory concentration; Control: Ascorbic acid.

extracts as promising natural source of antioxidants. Therefore, they can be used in nutritional or pharmaceutical areas for the prevention of free-radical-mediated diseases [52,53].

4. Conclusion

The present study determines that selecting endophytic fungi for various bioactivities represents a new source for obtaining novel bioactive metabolites with different potentialities. The statistical analysis of data is helpful in determining the endophytic biodiversity in a given plant species. Most of the isolates presented significant activity against pathogenic microorganisms. In particular, *A. macrospora, C. rosea* and *Fusarium* spp. showed promising antagonistic activity *in vitro* towards growth of the tested pathogens. These biocontrol profiles suggest that the above-mentioned endophytes indicate their potential as biotechnologically important cultures. Further studies are needed to improve our understanding of the complex plant-microbe interaction in this system. Potential endophytes from the present repository can be further used for studying plant growth promotion and extracellular enzymatic potential. Such studies would facilitate the selection of promising cultures possessing desirable properties and their exploitation in various biotechnological fields viz agriculture, industry and medicine.

Acknowledgements

Dr. Garima Gupta is acknowleged for her help in proof reading the manuscript.

Disclosure statement

The authors declare that they have no conflict of interests.

Funding

The authors are thankful to the the Department of Biotechnology, Govt. of India for financial assistance and facilities to conduct work on fungal endophytes of *Berberis aristata*. SK acknowledges DBT, Govt. of India for funding of the research project (BT/PR9538/NDB/39/425/2013). Bioinformatics Centre at School of Biotechnology and Central Instrumental Facility under FIST (DST, GOI) and SAP (UGC, New Delhi) programmes are also acknowledged.

References

[1] Freeman EM. The seed fungus of *Lolium temulentum* L., the darnel. Philos Trans R Soc Lond B. 1904;196:1–28.

[2] Sharma T, Kaul S, Dhar MK. Diversity of culturable bacterial endophytes of saffron in Kashmir, India. Springer Plus. 2015;4:661–674.

[3] Shahid M, Rahim T, Shahzad A, et al. Ethnobotanical studies on *Berberis aristata* DC. root extracts. Afr J Biotech. 2009;8:556–563.

[4] Lamichhane B, Adhikari S, Shrestha P, et al. Study of phytochemical, antioxidant, antimicrobial and anticancer activity of *Berberis aristata*. JTLS. 2014;4:01–07.

[5] Kumari K, Setty OH. The protective effect of *Berberis aristata* against mitochondrial dysfunction induced due to co-administration of mitomycin C and cisplatin. J Cancer Sci Ther. 2002;4:199–206.

[6] Tamilselvi S, Balasubramani SP, Venkatasubramanian P, et al. A review on the pharmacognosy and pharmacology of the herbals traded as Daruharidra. Int J Pharm Bio Sci. 2014;5:556–570.

[7] Pasrija A, Singh R, Katiyar CK. Comparative study on the antimicrobial activity of *Berberis aristata* from different regions and berberine *in vitro*. Int J Life Sci Res. 2011;1:17–20.

[8] Unkeshwar P, Nasiruddin M, Fayazuddin M, et al. Evaluation of hepatoprotective activity of *Berberis aristata* against carbon tetrachloride induced hepatotoxicity in rats. Int J Pharm Pharm Sci. 2013;5:254–265.

[9] Ramteke AD, Aiyer RB, Gandhi SP, et al. Phytochemical study of Daruharidra and hepatoprotective efficacy in infective hepatitis. J Pharm Sci Innov. 2013;1:44–47.

[10] Singh J, Kakkar P. Anti hyperglycemic and antioxidant effect of *Berberis aristata* root extract and its role in regulating carbohydrate metabolism in diabetic rats. J Ethnopharmacol. 2009;123:22–26.

[11] Dehar N, Walia R, Ratol S. Potentiation of thiopentone sodium induced hypnosis by *Berberis aristata* in rodents. Asian J Pharm Clin Res. 2013;5:131–133.

[12] Santos RMG, Rodrigues-Fo E, Rocha WC, et al. Endophytic fungi from *Melia azedarach*. World J Microbiol Biotechnol. 2013;19:767–770.

[13] Schulz B, Guske S, Dammann U, et al. Endophyte host interactions II. Defining symbiosis of the endophyte-host interaction. Symbiosis. 1998;25:213–227.

[14] Saghai-Maroof MA, Soliman KM, Jorgensen RA, et al. Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA. 1984;81:8014–8019.

[15] Li H, Qing C, Zhang Y, et al. Screening for endophytic fungi with antitumor and antifungal activities from Chinese medicinal plants. World J Microbiol Biotechnol. 2005;21:1515–1519.

[16] Schwalbe R, Steele-Moore L, Goodwin AC. Antimicrobial susceptibility testing protocol. Boca Raton, USA: CRC press; 2004.

[17] Orole OO, Adejumo TO. Activity of fungal endophytes against four maize wilt pathogens. Afr J Microbiol Res. 2009;3:969–973.

[18] Whipps JM. Developments in the biological control of soil borne plant pathogens. Adv Bot Res. 1997;26:1–134.

[19] Sanchez-Moreno C, Larrauri JA, Saura-Calixto F. A procedure to measure the antiradical efficiency of polyphenols. J Agric Food Chem. 1998;47: 425–431.
[20] Strobel GA. Endophytes as sources of bioactive products. Microbes Infect. 2003;5:535–544.
[21] Pandey A, Palni LMS. The rhizosphere effect in trees of the Indian Central Himalaya with special reference to altitude. App Ecol Env Res. 2007;5: 93–102.
[22] Kaul S, Ahmed M, Zargar K, et al. Prospecting endophytic fungal assemblage of *Digitalis lanata* Ehrh. (foxglove) as a novel source of digoxin: a cardiac glycoside. 3 Biotech. 2013;3: 335–340.
[23] Xu L, Wang J, Zhao J, et al. Beauvericin from the endophytic fungus, *Fusarium redolens*, isolated from *Dioscorea zingiberensis* and its antibacterial activity. Nat Prod Commun. 2010;5:811–814.
[24] Gond SK, Mishra A, Sharma VK, et al. Diversity and antimicrobial activity of endophytic fungi isolated from *Nyctanthes arbor- tristis*, a well-known medicinal plant of India. Mycoscience. 2012;53: 113–121.
[25] Qin JC, Zhang YM, Hu L, et al. Cytotoxic metabolites produced by *Alternaria* spp., an endophytic fungus isolated from *Ginkgo biloba*. Nat Prod Commun. 2009;4:1473–1476.
[26] Pillai TG, Jayaraj R. *Colletotrichum gloeosporioides*: a true endophyte of the endangered tree, *Cynometra travancorica* in the Western Ghats. J Plant Pathol Microb. 2015;6:5.
[27] Gupta S, Kaul S, Singh B, et al. Production of gentisyl alcohol from *Phoma herbarum* endophytic in *Curcuma longa* L. and its antagonistic activity towards leaf spot pathogen *Colletotrichum gloeosporioides*. Appl Biochem Biotechnol. 2016;180: 1–17.
[28] De Siqueira VM, Conti R, De Araujo JM, et al. Endophytic fungi from the medicinal plant *Lippia sidoides* Cham. and their antimicrobial activity. Symbiosis. 2011;3:89–95.
[29] Kaur M, Aggarwal NK. First record of *A. macrospora* MKP1 causing leaf spot disease on *Parthenium hysterophorus* from India. J Crop Prot. 2015;4:719–726.
[30] Bacon CW, White JFJ. Physiological adaptations in the evolution of endophytism in the Clavicipitaceae. Microb Endophytes. 2000;7:237–263.
[31] Kharwar RN, Verma VC, Kumar A, et al. Endophytic fungi: better players of biodiversity, stress tolerance, host protection and antimicrobial production. In: Chauhan AK, Verma A (eds) A textbook of molecular biotechnology. New Delhi: IK international Publishing house; 2009; p. 1033–1357.
[32] Verma VC, Kharwar RN, Gange A. Biosynthesis of antimicrobial silver nanoparticles by the endophytic fungus *Aspergillus clavatus*. Nanomedicine. 2010;5:33–40.
[33] Bezerra JD, Nascimento CC, Barbosa R, et al. Endophytic fungi from medicinal plant *Bauhinia forficata*: diversity and biotechnological potential. Braz J Microbiol. 2015;46:49–57.
[34] Patil MP, Patil RH, Maheshwari VL. Biological activities and identification of bioactive metabolite from endophytic *Aspergillus flavus* L7 isolated from *Aegle marmelos*. Curr Microbiol. 2015;71: 39–48.
[35] Qadri M, Johri S, Shah BA, et al. Identification and bioactive potential of endophytic fungi isolated from selected plants of the Western Himalayas. Springer Plus. 2013;2:8–17.
[36] Crozier J, Thomas SE, Aime MC, et al. Molecular characterization of fungal endophytic morphospecies isolated from stems and pods of *Theobroma cacao*. Plant Pathol. 2006;55:783–791.
[37] Castillo UF, Strobel GA, Ford EJ, et al. Munumbicins, wide-spectrum antibiotics produced by *Streptomyces* NRRL 30562 endophytic on *Kennedia nigriscans*. Microbiology. 2002;148: 2675–2685.
[38] Deshidi R, Devari S, Kushwaha M, et al. Isolation and quantification of alternariols from endophytic fungus, *Alternaria alternata*: LC-ESI-MS/MS analysis. Chemistry Select. 2017;2: 364–368.
[39] Lawrence R, Tripathi P, Jeyakumar E. Isolation, purification and evaluation of antibacterial agents from *Aloe vera*. Braz J Microbiol. 2009;40:906–915.
[40] Biswas B, Rogers K, McLaughlin F, et al. Antimicrobial activities of leaf extracts of guava (*Psidium guajava* L.) on two gram-negative and gram-positive bacteria. Int J Microbiol. 2013;2013:1–7.
[41] Kumar S, Upadhyay R, Aharwal RP, et al. Antibacterial activity of some isolated endophytic fungi from *Menthe viridis*. Int J App Biol Pharm Technol. 2016;7:239–248.
[42] Devaraju R, Satish S. Endophytic mycoflora of *Mirabilis jalapa* L. and studies on antimicrobial activity of its endophytic *Fusarium* spp. Asian J Biol Sci. 2011;2:75–79.
[43] Darshana CN, Praveena R, Ankegowda SJ, et al. Morphological variability, mycelial compatibility and fungicidal sensitivity of *Colletotrichum gloeosporioides* causing leaf spot of ginger (*Zingiber officinale* Rosc. J Spices Arom Crops. 2014;23: 211–223.
[44] Monkai J, Promputtha I, Kodsueb R, et al. Fungi on decaying leaves of *Magnolia liliifera* and *Cinnamomum iners* show litter fungi to be hyperdiverse. Mycosphere. 2013;4:292–301.
[45] Toledo AV, Virla E, Humber RA, et al. First record of *Clonostachys rosea* (Ascomycota: Hypocreales) as an entomopathogenic fungus of *Oncometopia tucumana* and *Sonesimia grossa* (Hemiptera: Cicadellidae) in Argentina. J Invertebr Pathol. 2006;92:7–10.
[46] Zhai MM, Qi FM, Li J, et al. Isolation of secondary metabolites from the soil-derived fungus *Clonostachys rosea* YRS-06, a biological control agent, and evaluation of antibacterial activity. J Agric Food Chem. 2016;64:2298–2306.
[47] Gong LJ, Guo SX. Endophytic fungi from *Dracaena combodiana* and *Aquilaria sinensis* and their antimicrobial activity. Afr J Biotechnol. 2009; 8:731–736.
[48] Motaal A, Nassar SM, Zayat SA, et al. Antifungal activity of endophytic fungi isolated from Egyptian henbane (*Hyoscyamus Muticus* L). Pak J Bot. 2010; 42:2883–2894.
[49] Rabha AJ, Naglot A, Sharma GD, et al. In vitro evaluation of antimicrobial activity of

Colletotrichum spp. against potent fungal pathogens of tea. Ind J Microbiol. 2014;54:302–309.

[50] Orlandelli RA, de Almeida TT, Alberto RN, et al. Antifungal and proteolytic activities of endophytic fungi isolated from *Piper hispidum* Sw. Braz J Microbiol. 2015;46:359–366.

[51] Yadav M, Yadav A, Yadav JP. In vitro antioxidant activity and total phenolic content of endophytic fungi isolated from *Eugenia jambolana* Lam. Asian Pac J Trop Med. 2014;7:256–261.

[52] Vasundhara M, Baranwal M, Kumar A. *Fusarium tricinctum*, an endophytic fungus exhibits cell growth inhibition and antioxidant activity. Ind J Microbiol. 2016;56:433–438.

[53] Tohidi B, Rahimmalek M, Arzani A. Essential oil composition, total phenolic, flavonoid contents, and antioxidant activity of *Thymus* species collected from different regions of Iran. Food Chem. 2017;220:153–161.