

Full Length Research Paper

Identification of endophytic fungi from roots of two *Dendrobium* species and evaluation of their antibacterial property

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Among all orchids, *Dendrobium* sp. is considered to have high medicinal value. *Dendrobium moniliforme* and *Dendrobium transparens* have immense pharmaceutical, commercial potentiality. However, their fungal endophytes remain unexplored. Isolation and identification of thirteen species of endophytic fungi from the root of *D. moniliforme* as well as five species from the roots of *D. transparens* were done. The two endophytic fungi namely *Aspergillus flavus* and *Trichoderma harzianum* were common for both plant species. Antimicrobial assay was done against selected human pathogen both gram positive and negative *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. The antimicrobial assay showed the significant role of endophytic fungi to inhibit the bacterial growth. Methanolic extract of DeMon VI and DeMon X were prepared to identify the bioactive compounds. Phenolic and 3-Eicosene,(E)-compounds were identified from extract of DeMon VI whereas Pentadecanoic acid, 14 methyle-, methyl ester and Diethyl Phthalate were identified from extract of DeMon X.

Key words: Orchids, *Dendrobium* sp., endophytic fungi, Human pathogen.

INTRODUCTION

Orchids are the most fascinating group of flowering with immense advantage and shows diversity in distribution (Pant and Raskoti, 2013; Pant et al., 2017). They are mostly found in moist and shady place, some are lithophytes, saprophytic and even terrestrial. These orchids have both ornamental as well as a medicinal value, used in traditional medicine to cure different diseases. Most of the orchids are listed on the Convention on International Trade in Endangered Species (CITES) Appendix II, its exploitation is still carried on (Pant and Raskoti, 2013). In Nepal, the orchid

are under threats and at verge of extinction (Subedi et al., 2013). The endophytic fungi resides in the plant tissue without causing harm to host plant and protect against pathogenic attacks by means of secondary metabolites (Schulz, 2006). Fungal endophyte provides variety of secondary bioactive products that have wide application in medicine, agriculture and industry (Selim et al., 2012).

Dendrobium is the second largest genus of orchid, about 30 species are found in Nepal. Most of these species have medicinal property but are verge of extinction. Recent report explores the antibacterial activity

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of the *Dendrobium* plant extract. In the present study, two *Dendrobium* species, *D. moniliforme* and *D. transparens* have been selected. These species are naturally found in central hills of Nepal. *D. moniliforme* are typical epiphytic orchid with white petal and semispherical anther (Xiaohua et al., 2009; Pant et al., 2016). The extract of *D. moniliforme* has significant role in curing osteoporosis and bone fracture (Baek et al., 2016). It has been used in traditional medicine. *D. transparens* is a typical epiphyte with graceful medium size flower. Flower has white petal with purple blotch at the tip (Sunitibala and Kishor, 2009). The medicinal properties of the *D. transparens* have not been studied yet. However, the reports suggest that it is used to cure various diseases by local people in least developed countries.

The multi drug resistance bacteria have become a threat to the public health and challenge to the scientific community. Over the years, human pathogenic bacteria have developed the mechanism to deal with various antibiotics. The multidrug resistant bacteria have become a global threat as these bacteria shows resistant to broad spectrum antibiotics. As a result, multiple drug resistant (MDR) bacteria spreads different diseases causing high mortality, high healthcare cost (Köck et al., 2010; Mongalo et al., 2013). Report suggest, the Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Acinetobacter baumannii* have developed the mechanism to resistant to most all antibiotics (Chambers, 1997; Kim et al., 2005; Patzer and Dzierzanowska, 2007).

This study highlights the isolation and identification of endophytic fungi from the roots of the selected *Dendrobium* species. The study focus on the antimicrobial activities of isolated and identified fungi against selected human pathogen. These studies explore the alternate method to tackle the multidrug resistant bacteria. In this regard, the human pathogen that were selected for antimicrobial assay are *E. coli*, *P. aeruginosa*, *S. aureus* and *Staphylococcus epidermidis*.

MATERIALS AND METHODS

The selected two *Dendrobium* species were collected from the two different places of the Central Nepal. *D. moniliforme* was collected from dense forest of Daman, Makwanpur district at an altitude of 1400-1600 m and the host tree was *Quercus semicarpifolia*. *D. transparens* was collected from the dense forest of Suryabinayak, Bhaktapur at an altitude of 1500-1600 m and the host tree was *Rhododendron* species.

Isolation of endophytic fungi from wild roots

A sterile scalpel was used to take the healthy roots from the three different epiphytic orchids. For the fungal isolation, method followed was described by Porras-Alfaro and Bayman (2007). First the roots were washed with running tap water for 20 min, 75% ethanol for 1 min, 3% Sodium hypochlorite (NaOCl) for 3 min and

rinsed with sterile water for 3 times. Then the roots were cut into 1 cm pieces. 5 pieces of roots were placed in 15 petri plates containing Potato dextrose agar (PDA) supplemented with antibiotics as chloramphenicol 50 μ g ml⁻¹ kept in room temperature (25-27°C) at dark for 3-7 days. The fungi growth were observed with their different colony pattern and were sub-cultured to obtain pure culture based on colony pattern. For the conservation of fungi, it was stored in freeze at 4°C until use.

Identification of fungi from root of selected wild *Dendrobium* species

Each pure colony obtained was used for the identification of fungal strain to genus and or species level on cultural characteristics and some morphological traits as overse and reverse colony colour, colony texture and the growth patterns. The microscopic characterization of strain was based on observation through microscope. Specimens for light microscope was mounted in lactophenol - cotton blue for observation of spores and sporulating structure and photograph were taken. Fungi were identified with the help of monographs, literature and following books: Watanabe 2010 and website Mycobank.org

Collection of bacterial strain

The bacterial strain used for the assay of antibacterial activities of orchid fungal endophytes was human pathogenic bacteria. *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) were gram negative bacteria whereas *S. aureus* (ATCC 259263) and *S. epidermidis* (ATCC 12228) were gram positive bacteria. These bacterial strains were identified and obtained from Shukraraaj Tropical and Infectious Disease Hospital, Kathmandu, Nepal.

Biochemical analysis of two fungal extract

Fungi were grown in 300 ml of Czapek broth for 15 days in the rotator shaker. The supernatant was obtained after centrifugation of the fungal broth at 5000 rpm for 30 min and then further filtered with the help of Whatman filter paper 1. The pH of the filtrate was made acidic by adding few drops of 5% sulphuric acid. Further, the three consecutive wash with equal volume of ethyl acetate was done. The organic portion, ethyl acetate extract was then collected with help of separating funnel. The residue of crude extract was obtained by subjecting the ethyl acetate extract to the rotary evaporator at 40°C. The residue was then suspended in 2 ml of High-performance liquid chromatography (HPLC) grade methanol. The bioactive compound was identified with the help of GCMS-QP2010 Ultra instrument fitted with RTX-5MS (30 m x 0.25 x 0.10) column. The various parameters was considered such as initial temperature of 100°C for 1 min and the final temperature of the oven 25°C. Rate of Helium flow of the instrument was 1 ml/min and the ionization voltage was 0.80 KV. The sample injection was in split less mode. Mass spectral scan range was set at 30 to 600 (m/z). The peak obtained for each compound in the graph was analyzed by comparing with the library of National Institute of Standard and Technology, NIST, US.

Antibacterial assay

The antibacterial test was performed by radial streaking method. In this regard, dual culture technique was followed. Each PDA plate consisting 1cm of fungi disc at center was incubated for three days at temperature of 25°C in dark condition and same plate was used for the antibacterial test. The different strain of bacteria

was taken which were streaked with the help of cotton swap from the periphery of plate towards center up to the end of colony of fungi. After swapping, the plates were kept in the incubator at 30°C for 24 h and results were observed. The experiment was repeated independently for three times. For the control, gentamycin disc of 10 mg was used, which was kept at the center of the petri plate and all the selected bacterial strain were streak from the radial ends of plate and incubated at 30°C for 24 h and the results were noted.

Measurement of zone of inhibition (ZOI)

After 24 h of incubation at 37°C, the plates were observed. The antibacterial test was evaluated as the presence or absence of inhibition zone. The clear distance between fungus and bacteria was zone of inhibition and measurement was taken in mm by the help of the scale. The width of inhibition zones between the pathogen and the endophytes was evaluated as >10 mm (strong inhibition), 2-10 mm (moderate inhibition) and <2 mm (weak inhibition) (Paul et al., 2007). For positive control, the inhibition zone was measured following same procedure as with fungi.

Statistical analysis

The results presented are the means of the three independent replicates \pm standard error of mean (S.E.M). The one way ANOVAs was done to find significant level of the data at the level of $P < 0.01$ and analyzed with the help of Microsoft Excel.

RESULTS

Identification of isolated fungi

Identification was carried out on the basis of macro-morphology and micro-morphology. Macro-morphology includes overse and reverse colony colour, colony texture and growth rate of colony whereas micro-morphological study includes conidia size, shape, mycelium, spore size and shape through photograph taken. Fungi were identified with the help of available literature, photograph and monograph method and spore structure was determined. A total of sixteen endophytic fungi species were isolated and identified, out of which thirteen strains were isolated from *D. moniliforme* and five strains from *D. transparens*. Interestingly, two species were common in both species. Most dominant species found was *Aspergillus* species and *Fusarium* species. *Aspergillus flavus*, *Aspergillus clavatum*, *Aspergillus brevipes*, *Aspergillus fumigatus*, *Hypoxylon fragiforme*, *Aspergillus niger*, *Colletotrichum* sp., *Leptosphaerulina chartarum*, *Fusarium* sp., *Cladosporium* sp., *Fusarium equiseti*, *Trichoderma harzianum* were identified from *Helminthosporium* sp., *Fusarium* sp., and one strain of isolated fungi from *D. transparens* remained unidentified (Figure 1 and Table 1).

Chemical profiling

The chemical profiling of the organic extract of two fungi was done. The organic extract of DeMon X and DeMonVI

showed the presence of diverse secondary metabolites. The major compound identified from the Gas Chromatography Mass Spectrometry (GC/MS) analytical technique from the extract of DeMonVI were Phenol, 2,4-bis(1,1-dimethylethyl)-, Eicosene, Pentadecanoic acid, 3-Octadecene. The major compound identified from the GCMS analytical technique from the extract of DeMon X were Diethyl Phthalate, Pentadecanoic acid, 14-methyl-, methyl ester. The detail of the chemical diversity of the both fungal extract is shown in the GC-MS chromatograph in supporting Figure 1a-p and Table 2.

Antimicrobial assay

The isolated and identified fungi from *D. moniliforme* and *D. transparens* showed antibacterial properties against at least one of four human pathogenic bacteria. All the endophytic fungi isolated from both the species of *Dendrobium* showed strong antibacterial activity against gram positive *S. epidermidis*. However, some endophytic fungi like *A. brevipes*, *A. fumigatus*, *Colletotrichum alatae*, *L. chartarum*, *Helminthosporium* species and *Fusarium* II species did have significant impact at level of $p < 0.01$ on growth inhibition against human pathogenic gram positive *S. aureus*.

Similarly, the endophytic fungi are tested against gram negative bacteria following same method. Almost all fungi isolated have highest zone of inhibition against *E. coli* except *Hypoxylon fragiforme*. Most of the fungi showed the growth inhibition of bacterium *P. aeruginosa* except endophytes *H. fragiforme*, *F. equiseti*. The positive control (gentamycin) showed moderate zone of inhibition against all four different human pathogenic bacteria.

There is significant growth inhibition at the significance level of $p < 0.01$ for the selected human pathogen by the orchid fungal endophytes summarized in Figure 2. The figure shows the comparative study of their pathogen growth inhibition activity among the endophytes and with respect to the control. The zone of inhibition of fungi was greater than control gentamycin (10 mg). The results of Table 2 depict the level of the inhibition against the four different bacteria. Nine species of fungi namely *A. flavus*, *A. clavatum*, *A. niger*, *Fusarium I* species, *Fusarium oxysporium*, *Cladosporium tenuissimum*, *Trichoderma*, *Fusarium II* species and one unidentified species have highest antibacterial properties with all four human pathogenic bacteria.

DISCUSSION

Most of the endophytic fungi isolated were *Aspergillus* sp., *Fusarium* sp., *Trichoderma* sp., *Hypoxylon* sp., *Colletotrichum* sp., *Leptosphaerulina* sp., *Cladosporium* sp., and *Helminthosporium* sp. belonging to ascomycetes. *Aspergillus* sp. has been well known for its medicinal

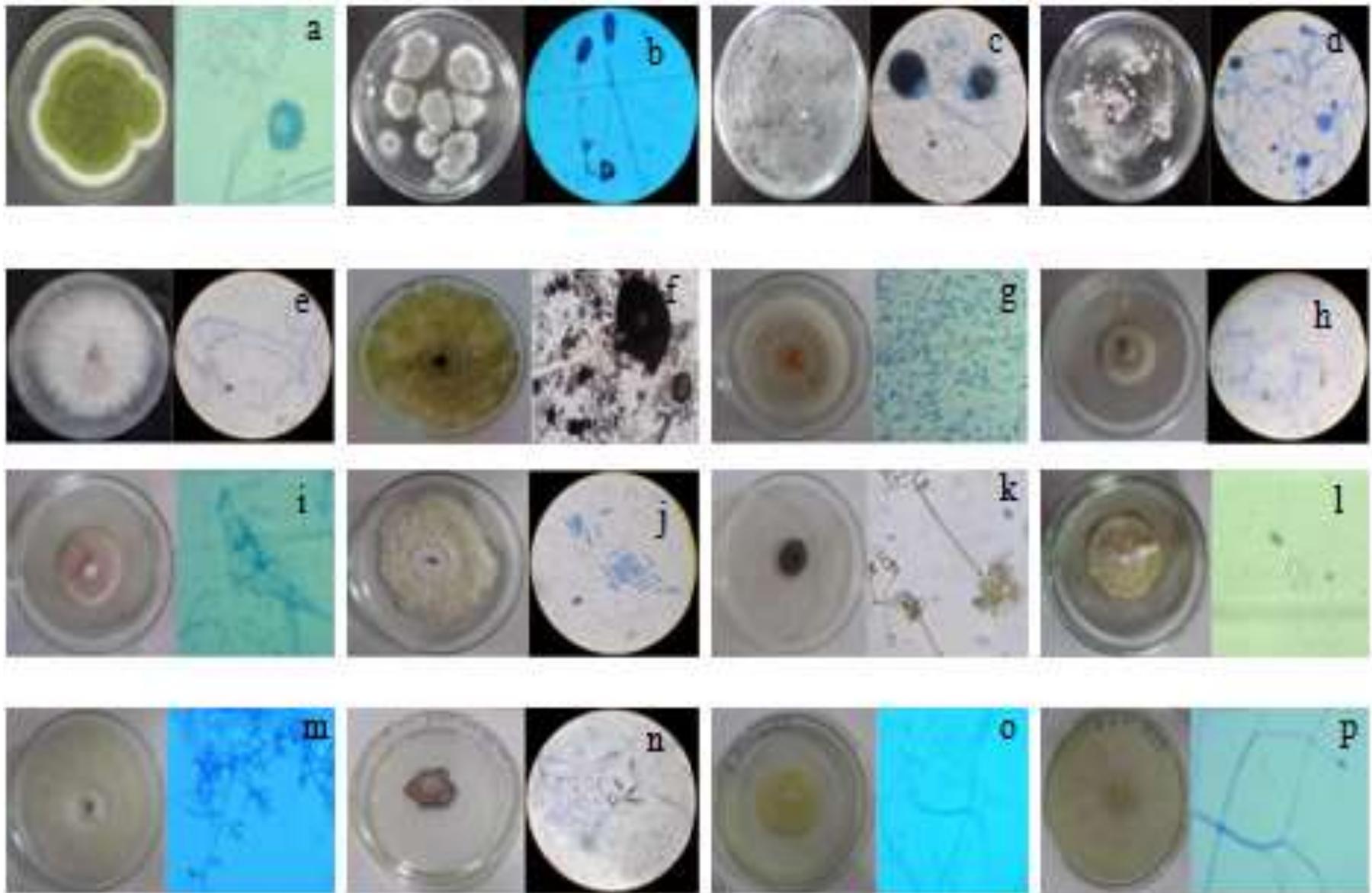


Figure 1 (a-p). Over-se colony on PDA and microscopic view at 40X of endophytic fungi. (a) *Aspergillus flavus*, (b) *A. clavatum*, (c) *A. brevipes*, (d) *A. fumigatus*, (e) *Hypoxylon* sp., (f) *A. niger*, (g) *Colleotrichium* sp., (h) *Leptosphaerulina* sp., (i) *Fusarium* sp., (j) *Fusarium* sp., (k) *Cladosporium* sp., (l) *Fusarium equiseti*, (m) *Trichoderma* sp., (n) *Helminthosporium* sp., (o) *Fusarium* sp., and (p) unidentified respectively.

Table 1. List of endophytic fungi from roots of selected wild *Dendrobium* species.

Code No. of isolates	Peculiar Characteristics of isolates	Tentative affiliation	Fungal taxonomy
DeMon I/DeTra V	Colony radial growth with grey to yellowish colour. Conidial head radiate and biseriate with globose shaped	<i>Aspergillus flavus</i>	Eurotiomycetes
DeMon III	Colony radial growth with grey to green, Conidial head radiate and uniseriate with ellipsoidal	<i>Aspergillus clavatum</i>	Eurotiomycetes
DeMon IV	Colony radial growth with grey to green colour. Conidial head uniseriate and columnar with globose shaped	<i>Aspergillus brevipes</i>	Eurotiomycetes
DeMon V	Colony radial growth with white to greenish on maturation. Conidial head very short with uniseriate and vesicle globose shaped	<i>Aspergillus fumigatus</i>	Eurotiomycetes
DeMon VI	Colony radial growth with white to black on maturation. The mycelium was observed which were branched and septate no sporulating stage	<i>Hypoxylon</i> sp.	Euascomycotina
DeMon VII	Colony radial growth with was brown to black on maturation. Conidial head were biseriate and globose in shape, Vesicles were spherical to globose	<i>Aspergillus niger</i>	Eurotiomycetes
DeMon X	Colony radial growth with white to pale orange at the centre on maturation. Conidia were cylindrical with broadly rounded ends	<i>Colletotrichum</i> sp.	Sordariomycetes
DeMon XI	Colony radial growth with grayish and become brown. Mycelium was brown and septate.	<i>Leptosphaerulina</i> sp.	Dothideomycetes
DeMon XII/XVII	Colony radial growth with white at first and become pink. Conidia are smaller in size and are cylindrical, gradually pointed and curved towards end.	<i>Fusarium</i> sp.	Sodariomycetes
DeMon XIII	Colony radial growth with white to pinkish. Conidia was comparatively larger in size gradually pointed and curved towards ends	<i>Fusarium oxysporium</i>	Sodariomycetes
DeMon XIV	Colony radial growth with greenish brown on maturation. The hyphae was brown, erect, and septate. Conidiophores was brown may be septate and shows tree like branching	<i>Cladosporium</i> sp.	Dothideomycetes
DeMon XVI	Colony radial growth with white to greenish on maturation. The conidia are curvature, tapered and elongated apical cell	<i>Fusarium equesti</i>	Sordariomycetes
DeMon XIX/DeTra II	Colony radial growth with white to greenish on maturation. Conidial were globose to sub-globose, flask shaped and arranged in divergent groups	<i>Trichoderma</i> sp.	Sordariomycetes
DeTra I	Colony radial growth with grayish brown on maturation. Hyphae was branched, septate, pale brown. Conidiophores was cylindrcial and curvature	<i>Helminthosporium</i> sp.	Dothideomycetes
DeTra III/IV	Colony radial growth with white to pale yellow on maturation. microconidia are cylindrical in shape	<i>Fusarium</i> sp.	Sordariomycetes
DeTra VI/VII	Colony radial growth with white cottony to pale yellow on maturation. Mycelium baranched aseptate Conidiophore oval or globose shaped	Unidentified	Unknown

oactive compound importance and that have been catalogued as a scientific database (Furtado et al., 2002; Thorati and Mishra, 2017). The most important and well-known compound isocoumarin 7,7'-homodimers and dihydroquinolone derivative have been isolated from *A. versicolor*. The report clearly demonstrates the biological activities such as antimicrobial as well as cytotoxicity. In the present study, *A. fumigatus* and *A. niger* that has

been isolated and identified were also studied for their antimicrobial activity in the previous study (Furtado et al., 2002; Thorati and Mishra, 2017). *A. fumigatus* is known to produce 3,4-dimethoxyphenol and 1,3,5-trimethoxybenzene (Furtado et al., 2002). In our findings, the *Hypoxylon* sp. was able to inhibit the growth of two pathogen *S. aureus* and *S. epidermidis*. However, the fungi did not show any effect on the growth of *E. coli* and

Table 2. List of the various bioactive compounds identified from fungal extract of DeMon X and DeMon VI.

Peak	Retention time	Area%	Name	Base m/z	Reported biological activities
1	6.261	5.02	Phenol, 2,4-bis(1,1-dimethylethyl)-	191.05	Antibacterial, antifungal and antioxidant properties (Ramanujam et al., 2014)
2	6.823	20.33	3-Octadecene, (E)-	43.05	Antimicrobial property (Guo et al., 2008)
3	7.002	13.10	Diethyl Phthalate	149.25	Antimicrobial property
4	8.307	16.52	3-Eicosene,(E)-	43.10	(Dehpour et al., 2012)
5	9.229	3.09	Petadecanoic acid, methyle-, methyl ester	14 74.00	Antimicrobial property
6	9.260	45.84	Petadecanoic acid, methyle-, methyl ester	14 74.15	(Dehpour et al., 2012)

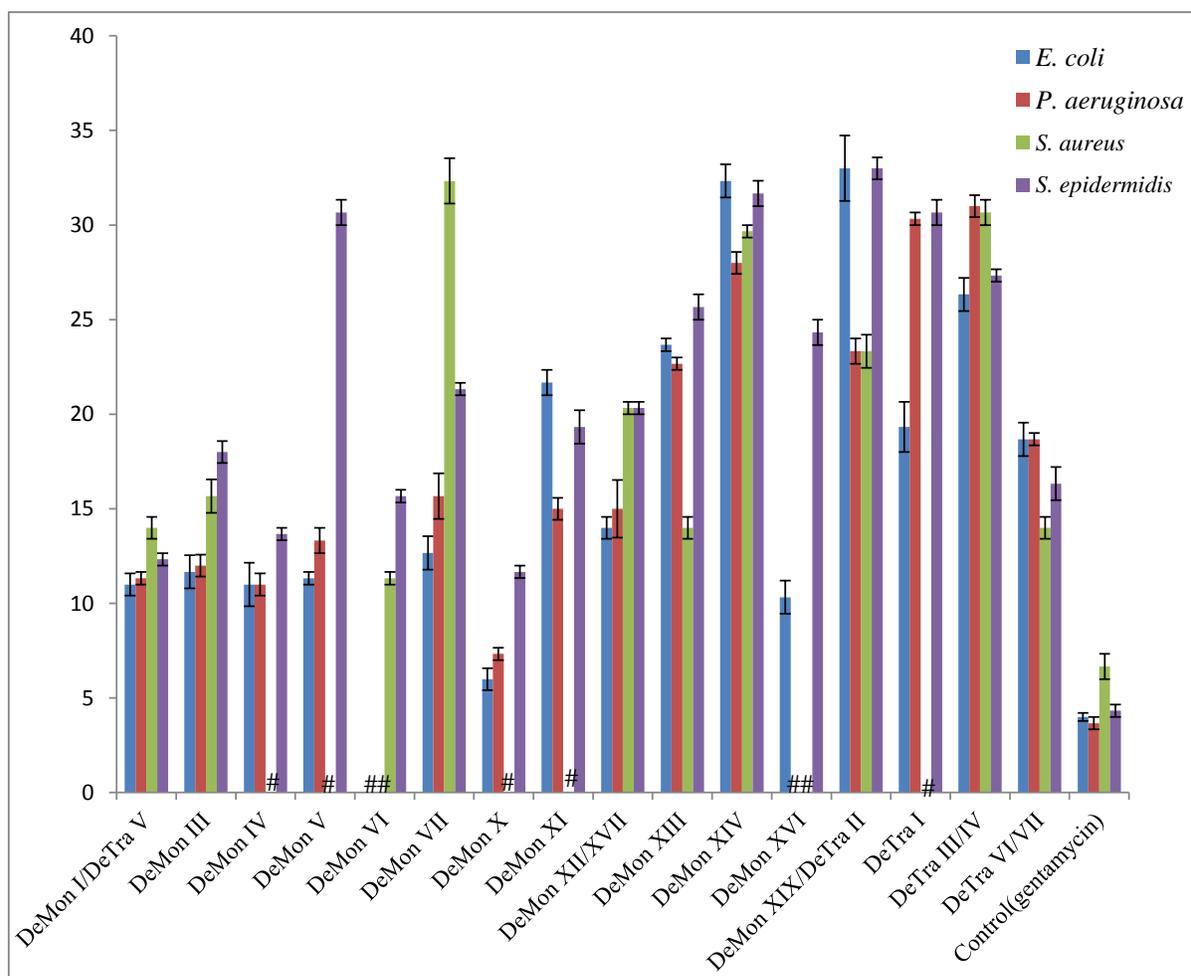


Figure 2. Antibacterial activity of the endophytic fungi against selected humane pathogen. The fungi DeMonIV, DeMon XIX, DeMon XII, DeMonXVII showing high growth inhibition of the pathogen compared to antibacterial activity of other fungi and control.# represent no antibacterial activity of the corresponding fungus. One way ANOVAs was performed for the significant level of $p < 0.01$.

P. aeruginosa. The chemical profiling of the *Hypoxylon* sp. DeMon VI showed the presence of Phenol, 2,4-bis(1,1-dimethylethyl)-, Eicosene, Pentadecanoic acid, 3-

Octadecene. The compound has been well investigated for their antioxidant and antibacterial activities (Dehpour et al., 2012). Similarly, *Colletotrichum* sp., also

demonstrate the antimicrobial properties. The organic extract of DeMon X was investigated for bioactive compound. The compounds such as Diethyl Phthalate, Pentadecanoic acid, 14-methyl-, methyl ester were identified from extract. These compounds have both antimicrobial and antioxidant activities. However, the fungus appears to show antibacterial activities but was ineffective against *S. aureus*. Similarly, DeMon XI isolated from *Dendrobium moniliforme* have been characterized as *Leptosphaerulina* sp. The DeMonXI was able to show the antibacterial effect against *E. coli*, *P. aeruginosa*, *S. epidermidis* but was ineffective against *S. aureus*. However, similar kind of study was done with Endophytic fungi *Leptosphaerulina* sp. isolated from the mangrove plant *Acanthus ilicifolius*. The fungus was reported to produce novel compounds pyranonaphthazarin and 2-naphthoic acid derivatives having strong antimicrobial properties against *S. aureus* (Cui et al., 2017). DeMon XIV was characterized as *Cladosporium* sp. that has shown strong antibacterial activity against all the selected human pathogenic bacteria. In previous study, Phenylacetic acid, p-hydroxyphenylethyl alcohol, and L-beta-phenyllactic DeMonXI was acid were isolated from the extract of *Cladosporium* sp. that has an antimicrobial property (Ding et al., 2008). The result correlates the presence of such metabolites that have shown higher antibacterial activity in our investigation.

In the present study, *Fusarium* species were relatively isolated in large number. Most of them are known for their antimicrobial, antioxidant activities as well as secondary metabolite production. DeMonXII, DeMonXIII, DeMonXVII and DeMonXVI isolated from *D. moniliforme* and DeTra III and DeTra IV isolated from *D. transparentis*. These fungi were characterized as *Fusarium* sp. and were able to show antibacterial activity against the selected human pathogen. DeMon XII, DeMonXIII, DeMon XVII, DeTra III and DeTra IV strongly inhibited the growth of all four selected human pathogenic bacteria. However, the isolate DeMonXVI show the activity against *E. coli* and *S. epidermidis*. Similarly, DeMon XIX and DeTra I were characterized as *Trichoderma* species and were able to strongly inhibit the growth of all four selected human pathogenic bacteria. Importantly, a novel L-lysine oxidase enzyme have identified from the fungal extract of *Trichoderma viride* that have antitumor activity. New species of *Trichoderma hypoxylon* produces various secondary metabolites such as trichothecenes and epipolythiodiketopiperazines (Sun et al., 2016). Similarly, the various secondary metabolites have been reported from *Trichoderma* species may have contributed to their antibacterial activity. DeTra I which is characterized as *Helminthosporium* species was able to inhibit the growth of all three human pathogens *E. coli*, *P. aeruginosa*, and *S. epidermidis* except *S. aureus*. The fungi that remain uncharacterized by macro and micro morphology also showed that significant antibacterial activity against all the human pathogenic bacteria.

Conclusion

This research concludes that large number of fungi belonging to the phylum ascomycetes is reside in the roots of wild *D. moniliforme* and *D. transparentis* species. On the basis of morphological study sixteen species of fungi were identified. Among them thirteen species from *D. moniliforme* and five species from *D. transparentis* and two species common in both species. The identified fungi were tested against four different human pathogenic bacteria *E. coli*, *P. aeruginosa*, *S. aureus* and *S. epidermidis*. Each fungus shows antibacterial properties against at least one of the four human pathogenic bacteria. Most of the fungi show antibacterial activity against *S. epidermidis*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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