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Original Article

ANTIMICROBIAL POTENTIAL OF FUNGAL ENDOPHYTES ISOLATED FROM LEAVES OF *PROSOPIS JULIFLORA* (SW.) DC. AN IMPORTANT WEED

APARNA SRIVASTAVA[1]*, RAVEESHA KOTESHWAR ANANDRAO[2]

Centre for Innovative Studies in Herbal Drug Technology, Department of Studies in Botany, University of Mysore, Manasagangotri, Mysuru 570006
Email: karaveesha@gmail.com

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ABSTRACT

Objective: To evaluate the antimicrobial potential of fungal endophytes isolated from leaves of *Prosopis juliflora* against the important plant and human pathogenic bacteria and fungi.

Methods: Antimicrobial screening was done by agar diffusion and dual culture method. The ethyl acetate extract of the endophytes which recorded good activity were subjected to disc diffusion assay and determination of MIC.

Results: A total of 446 fungal isolates were recovered from 400 leaflet segments representing 44 fungal endophytes. Species of *Cladosporium*, *Colletotrichum* and *Fusarium* were the dominant genera. Seventeen endophytic fungi out of 44 showed significant antibacterial activity against one or more test bacteria. Ethyl acetate extract of *Colletotrichum gloeosporioides* exhibited highly significant broad spectrum antibacterial activity. Eleven endophytes showed antifungal activity against all test fungi. *Paecilomyces lilacinus* and *Trichoderma* sp. showed significant antifungal activity with growth inhibition of 50% and above in dual culture. The ethyl acetate extract of *Paecilomyces lilacinus* also showed significant antifungal activity in disc diffusion. This is the first report of antimicrobial potential of the endophyte *Paecilomyces lilacinus*. Preliminary biochemical characterization of the active ethyl acetate extract of *Colletotrichum gloeosporioides* and *Paecilomyces lilacinus* showed the presence of alkaloids, carbohydrates, sterols and coumarins.

Conclusion: Results of the study paves the way for further studies on the isolation and characterization of antimicrobial principles and their evaluation for the agricultural and medical application.

Keywords: Antimicrobial activity, Biochemical screening, *Colletotrichum gloeosporioides,* Fungal endophytes, *Paecilomyces lilacinus*.

INTRODUCTION

The discovery of microbe-produced bioactive compounds and their utilization in health care has intensified the stream of attention to endophytes as a big reservoir of especially inhabiting microorganisms [1]. Endophytes are microbes residing in healthy plant tissues without causing any negative impact on the host plants [2]. Studies have demonstrated that endophytes occupy millions of unique ecological niches in many unusual environments [3, 4] and provide specific advantages of protecting the host against insects-pests, pathogens and even domestic herbivores [5, 6].

More than 8600 biologically active compounds have been reported from fungi with various usages such as antimicrobial, antiviral, anticancer, antidiabetic, etc. [3, 4, 7, 8]. In some cases, plant-associated fungi are able to make the same bioactive compound as the host plant itself. The discovery of gibberellins in *Fusarium fujikuroi* and Taxol from the endophytic fungi, *Taxomyces andreanae* associated with *Taxus brevifolia* [9] are good examples. Reports are available on isolation and diversity of endophytic mycoflora from Indian medicinal plants such as *Azadirachta indica, Aegle marmelos, Terminalia arjuna*, *Adhatoda zeylanica, Adenocalymma alliaceum*, *Ocimum sanctum, Adhathoda vasica, Withania somnifera, Cannabis sativa* and *Viola odorata* [7, 10-13]. Endophyte association with weeds like *Mikania micrantha*, *Chenopodium album, Euphorbia helioscopia, Parthenium hysterophorus, Convolvulus arvensis* and *Monochoria vaginalis* are also reported [14-16]. According to Strobel [17], plants with medicinal value or unusual longevity, the plants that survive under extreme conditions often harbor potential fungal endophytes that produce bioactive metabolites.

A considerable number of antimicrobial compounds have been isolated from the endophytic fungi of *Artemisia mongolica, Artemisia annua, Juniperus cedre, Sonneratia alba, Azadirachta indica*, *Vitex negundo*, *Lippia sidoides*, *Brassica napus, Plumeria acuminata* and *Plumeria obtusifolia* [18-26]. Thus, endophytes hold tremendous promise as an alternative source for producing valuable bioactive compounds with varied applications, both in research and applied fields of medicine, agriculture, food industry and pest management.

Prosopis juliflora is an important weed plant with unique biology and it has the ability to survive in varied environmental conditions. The plant possesses a large number of secondary metabolites which are produced from different parts of the plant. It is found to contain alkaloids, flavonoids, terpenoids, tannins, sugars and amino acids. These secondary metabolites of the plant are reported to possess good antimicrobial, anti-inflammatory, anti-malarial and also growth inhibitory activities [27]. The different groups of alkaloids isolated from this plant are reported to possess significant antibacterial and antifungal activity which is comparable to the standard drugs [28, 29]. Antifungal potential of the alkaloid fraction of the leaves of *P. juliflora* against important pathogens has been reported, and its utilization for the management of seed borne crop diseases has been established from this lab [30].

Thus, the aim of this study was to isolate and characterize the fungal endophytes associated with leaves of *P. Juliflora* and to evaluate their potential as antibacterial and antifungal agents against a range of pathogenic bacteria and fungi. Since, this is the maiden report of endophytic mycoflora of *P. Juliflora* with their antimicrobial activity; we hope these initial steps of product discovery will pave a way for discovering new bioactive principles from the endophytes of this plant.

MATERIALS AND METHODS

Isolation of fungal endophytes

Healthy leaves of *Prosopis juliflora* were collected from the surroundings of Kukarahalli Lake, Manasagangotri, Mysuru, India (12.30 ° N 76.65 ° E). Collection was made in sterile polythene bags and transported to the laboratory. Leaves were washed thoroughly in running tap water for 10 min to remove debris. Surface sterilization was done by adopting the methodology of Petrini [2],

and the effectiveness of the same was checked following the procedures of Schulz [3]. Epiphytic mycelia were removed by immersing the tissues in 70% ethanol for 1 min and in the aqueous solution of sodium hypochlorite (4% available chlorine) for 3 min followed by washing with 70% ethanol for 5-10 s. Finally, the leaves were rinsed three times in sterile distilled water and dried with a sterile paper towel.

After surface sterilization, leaves were cut into very small segments of approximately 2X2 mm using a flame-sterilized scalpel and placed on Potato Dextrose Agar (PDA) (supplemented with chloramphenicol (200 mg/l) to suppress the growth of bacteria) such that freshly cut edges come in direct contact with the potato dextrose agar surface.

The plates were regularly observed for fungal growth, and actively growing fungal tips emerging from leaflets were sub-cultured on PDA medium for identification and enumeration. The endophytic fungi were identified according to their macro and microscopic structures. Species-level identification was done by using standard manuals. All the isolated and identified, endophytic fungi were maintained on PDA slants layered with glycerol (15%, v/v) for its preservation.

Test pathogens

The isolated endophytic fungi were evaluated for their antimicrobial activity against the plant and human pathogenic bacteria and fungi. The test plant pathogenic bacteria were *Xanthomonas campestris* pv. *vesicatoria, Xanthomonas oryzae, Pseudomonas syringae* and *Ralstonia solanacearum,* isolated from diseased plant material. The test human pathogenic bacteria were *Bacillus subtilis* (MTCC121), *Bacillus cereus* (MTCC1272), *Escherichia coli* (MTCC7410) and *Staphylococcus aureus* (MTCC7433) obtained from IMTECH, Chandigarh, India.

The antifungal activity studies were conducted against *Fusarium solani, Fusarium verticillioides, Aspergillus flavus* and *Aspergillus ochraceus* isolated from Maize seeds.

Antimicrobial activity

Agar diffusion method

The inhibition of bacterial growth by endophytic fungi was examined on Nutrient Agar (NA) plates using agar diffusion method [31, 4]. Six-millimeter diameter mycelial plugs of actively growing endophytic fungi were placed in the NA media plate inoculated with test bacteria. The plates were incubated for 24-48 h at 37 ^{o}C. After 24 h, the zone of inhibition (ZOI) if any was measured in mm. These measurements were interpreted according to the following categories (+++):>20 mm ZOI as high activity, (++): 10-20 mm ZOI as moderate activity and (+):<10 mm ZOI as low activity [32].

Dual culture method

Endophytic fungi and fungal phytopathogens were cultured separately on PDA plate and incubated at 25±2 ^{o}C for 5 d. The growing fungal mycelium was cut by using 6 mm diameter cork borer. Fungal discs of endophytic fungi and fungal phytopathogens were placed on the opposite side of the same PDA plate, at a distance of 5 cm between the fungal pair. A fungal disc of plant pathogenic fungi placed on one side on a PDA plate without endophytic fungi was served as control. The dual cultures were incubated at 25±2 ^{o}C for 5 d. The fungal growth was determined by the radial growth of fungal phytopathogens and the percentage of inhibition was calculated by the following formula.

$$\text{Percentage of inhibition} = [(R1-R2)/R1] \times 100$$

Where R1 represents radial growth of fungal phytopathogens in control plate and R2 is the radial growth of fungal phytopathogens in test sample plate. The percentage of inhibition was categorized on a growth inhibition category level, from low to high antifungal activity; (+): < 20% = low antifungal activity, (++): 20-< 50% = moderate antifungal activity, (+++): 50-<70% = high antifungal activity, (-): No antifungal activity. The endophytic fungus which recorded more than 30% inhibition were selected for further work on the extraction of secondary metabolite and to assess the antifungal potential [32].

Fermentation and extraction of secondary metabolites

The endophytic fungi were grown in 500 ml of Erlenmeyer flasks containing 250 ml of potato dextrose broth and incubated at 25±2 ^{o}C on a rotary shaker at 150 rpm. After 2 d, 100 ml of liquid culture was transferred as a seed into one-litre flask containing 500 ml of potato dextrose broth for 21 d in stationary phase. The fermented broths were filtered through Whatmann No. 1 filter paper to separate the fungal mats and the culture filtrates. The culture filtrates were extracted twice with equal volume of ethyl acetate using a separating funnel. The culture filtrate extracts were concentrated using a rotary flash evaporator to yield ethyl acetate extract. Crude metabolites were weighed and finally dissolved in 2 ml of methanol. It was filtered through a filter paper to remove residues that do not dissolve in methanol and then the filtrate was diluted with methanol to obtain the concentration of 100 mg/ml for further use [3].

Disc diffusion method

Endophytic fungi which showed high antibacterial and antifungal activity in the preliminary assay were subjected to disc diffusion assay [33]. Sterile discs of 6 mm diameter were impregnated with 50 µl of ethyl acetate extracts of endophytic fungi using a micropipette and kept under a laminar hood for 20 min to dryness. Air-dried sterile discs impregnated with 100 mg of the ethyl acetate extracts were used to test the activity against selected test bacteria and fungi. The test culture was swabbed evenly with a cotton swab onto the surface of solidified Mueller-Hinton (MH) agar plates for bacteria and Czapek Dox Agar (CDA) plates for fungi. The discs containing 100 mg ethyl acetate extract were placed on the surface of the respective medium seeded with test cultures in separate petri plates. The disc impregnated with a respective solvent of the same volume served as control. The plates were incubated at 37±2 ^{o}C for 24 h for bacteria and 25±2 ^{o}C for 7 d for fungi and the zone of inhibition was measured after the incubation period. Each test was done in three replicates and mean and standard error was calculated. The analysis of variance (ANOVA) using statistical software IBM SPSS statistics for windows, version 20 to detect the significance of differences (p = 0.05) among different treatment means using Student-Newman-Keuls test.

Minimal inhibitory concentration

The minimal inhibitory concentration (MIC) value was determined for the ethyl acetate extract of *Paecilomyces lilacinus* and *Colletotrichum gloeosporioides* by serial plate dilution assay in accordance with the Clinical and Laboratory Standard Institute (CLSI) methodology [34]. MIC value was taken as the lowest concentration of the extract that inhibited any visible growth of the pathogen after incubation. The initial ethyl acetate extract concentration taken was 10 mg/ml and 2-fold serial dilution was done. It was tested at the concentration range from 5000 µg/ml to 2 µg/ml. 100 µl of Mueller-Hinton broth for bacteria and Czapek dox broth for fungi was pipetted into each well, to this 100 µl of the test pathogens suspension was then added. The organic solvents used for extraction served as negative control, in order to monitor sample sterility and to determine any antimicrobial effect of the solvents. The standard drugs viz. Neomycin, Gentamycin, and Carbendazim were used as positive control respectively. The microplates were incubated overnight at 37 ^{o}C, 24 h for bacteria and 25±2 ^{o}C, 7 d for fungi. After incubation, the optical density was measured to check the inhibition at 640 nm for bacteria and 590 nm for fungi. The clear solution indicated inhibition of test pathogens. Three replicates were maintained per test bacteria and fungi. The resultant MIC values were determined as the mean of these replicate experiments.

Biochemical screening

Ethyl acetate extracts of *Paecilomyces lilacinus* and *Colletotrichum gloeosporioides* were subjected to preliminary screening for the presence of active secondary metabolites, following standard procedures [35, 36]. Visible color change or precipitate formation revealed the presence (+) or absence (–) of particular active constituents.

RESULTS

Isolation of fungal endophytes

The efficiency of surface sterilization was confirmed by the lack of microbial growth in controls even after 30 d of inoculation. A fungal outgrowth from the cut ends of leaf tissues was first observed after 48 h of inoculation. A total of forty-four fungal endophytes were isolated from 400 leaflet segments of *P. Juliflora* is represented in table 1. In terms of total isolates under all endophytic taxa, 446 isolates were recovered. The maximum number of endophytic fungi belonged to Hyphomycetes (30) followed by Coelomycetes (6), Ascomycetes (6) and *Mycelia sterilia* (2). Zygomycetes and Basidiomycetes were absent.

Antimicrobial activity

Agar diffusion test

The fungal endophytes isolated from leaves of *P. juliflora* showed significant antibacterial activity against the test bacteria. Among 44 endophytic fungi, 17 showed antibacterial activity in agar diffusion method against the test bacteria. *Colletotrichum gloeosporioides* and *Fusarium moniliforme* showed strong inhibition against *Xanthomonas vesicatoria, Pseudomonas syringae, Bacillus cereus* and *Bacillus subtilis* with an inhibition zone of more than 20 mm diameter. *Alternaria alternata*, *Fusarium oxysporum*, *Colletotrichum gloeosporioides, Fusarium moniliforme* and *Penicillium* sp. showed moderate activity against one or more test bacteria with an inhibition zone of 10-20 mm diameter. *Alternaria* sp. and *Botryderma* sp. showed low antibacterial activity against *Xanthomonas vesicatoria* whereas sterile mycelium (hyaline) showed antibacterial activity against *Pseudomonas syringae* only, with an inhibition zone of less than 10 mm diameter as shown in table 1.

Dual culture method

Out of 44 fungal endophytes isolated from *P. Juliflora*, 11 fungal endophytes inhibited the growth of at least one of the fungal phytopathogens at the level of more than 20 % inhibition in the dual culture assay. *Paecilomyces lilacinus* and *Trichoderma* sp. showed significant antifungal activity with growth inhibition of more than 50 % against *Fusarium solani*, *Fusarium verticillioides, Aspergillus flavus* and *Aspergillus ochraceus*.

Table 1: Antibacterial activity of fungal endophytes against plant and human pathogenic bacteria by agar diffusion assay

S. No.	Endophytic fungi	Test bacteria			
		Xv	Ps	Bs	Bc
1	*Acremonium* sp.	-	-	-	-
2	*Alternaria brassicicola*	-	-	-	-
3	*Alternaria alternata*	++	++	+	++
4	*Alternaria* sp.	+	-	-	-
5	*Aspergillus terreus*	-	-	-	-
6	*Bipolaris bicolor*	-	-	-	-
7	*Bipolaris spicifera*	+	+	+	+
8	*Blastomyces* sp.	-	-	-	-
9	*Botrytis* sp.	-	-	-	-
10	*Botryderma* sp.	+	-	-	-
11	*Cladosporium acalyphae*	-	-	-	-
12	*Cladosporium oxysporum*	+	+	+	+
13	*Cladosporium herbarum*	-	-	-	-
14	*Cladosporium cladosporioides*	-	-	-	-
15	*Cladosporium sphaerospermum*	-	-	-	-
16	*Cladosporium* sp.	+	+	+	+
17	*Colletotrichum guajavae*	-	-	-	-
18	*Colletotrichum lindemuthianum*	-	-	-	-
19	*Colletotrichum gloeosporioides*	+++	++	+++	+++
20	*Colletotrichum tropicale*	+	+	+	+
21	*Colletotrichum* sp.	+	+	+	+
22	*Curvularia* sp.	-	-	-	-
23	*Cylindrocarpon* sp.	-	-	-	-
24	*Diplodina* sp.	-	-	-	-
25	*Fusarium moniliforme*	+++	+	++	+++
26	*Fusarium oxysporum*	+	++	+	+
27	*Helminthosporium dematioidae*	-	-	-	-
28	*Myrothecium* sp.	-	-	-	-
29	*Nigrospora* sp.	-	-	-	-
30	*Paecilomyces lilacinus*	+	+	-	-
31	*Penicillium* sp.1	+	+	+	+
32	*Penicillium* sp.2	++	+	+	+
33	*Phoma lingam*	-	-	-	-
34	*Phomopsis archeri*	+	+	+	+
35	*Pseudotorula* sp.	-	-	-	-
36	*Pestialopsis* sp.	-	-	-	-
37	*Pythiopsis* sp.	-	-	-	-
38	*Trichocladium* sp.	-	-	-	-
39	*Trichoderma harzianum*	-	-	-	-
40	*Trichoderma* sp.	+	+	+	+
41	*Verticillium* sp.	+	+	+	+
42	*Xylaria* sp.	-	-	-	-
43	Sterile mycelium (hyaline)	-	+	-	-
44	Sterile mycelium (narrow width)	-	-	-	-

Test bacteria: Xv-*Xanthomonas vesicatoria*; Ps-*Pseudomonas syringae*; Bc-*Bacillus cereus*; Bs-*Bacillus subtilis*

–: no inhibition zone (No activity), **+:** inhibition zone is less than 10 mm (Low activity), **++:** inhibition zone is from 10 mm to 20 mm (Moderate activity), **+++:** inhibition zone is above 20 mm (High activity).

The endophytes *Alternaria alternata*, *Botrytis* sp., *Colletotrichum gloeosporioides, Verticillium* sp., *Penicillium* sp., *Phomopsis archeri* and sterile mycelium showed moderate antifungal activity by inhibiting the growth between 20-50% against *Fusarium solani* and *Fusarium verticillioides* but did not show any activity against *Aspergillus flavus* and *Aspergillus ochraceus*. The endophyte *Fusarium oxysporum* showed significant activity against *Aspergillus ochraceus* only, whereas *Aspergillus terreus* showed significant activity against *Aspergillus ochraceus* and *Fusarium verticillioides*. The percentage of inhibition was presented in table 2.

Table 2: Antifungal activity of fungal endophytes against phytopathogenic fungi by dual culture method

S. No	Endophytic fungi	Test fungi			
		Fs	Fv	Af	Ao
1	*Acremonium* sp.	-	-	-	-
2	*Alternaria brassicicola*	-	-	-	-
3	*Alternaria alternata*	++	++	-	-
4	*Alternaria* sp.	-	-	-	-
5	*Aspergillus terreus*	++	+	-	-
6	*Bipolaris bicolor*	-	-	-	-
7	*Bipolaris spicifera*	-	-	-	-
8	*Blastomyces* sp.	-	-	-	-
9	*Botrytis* sp.	++	+	-	-
10	*Botryderma* sp.	-	-	-	-
11	*Cladosporium acalyphae*	-	-	-	-
12	*Cladosporium oxysporum*	-	-	-	-
13	*Cladosporium herbarum*	-	-	-	-
14	*Cladosporium cladosporioides*	-	-	-	-
15	*Cladosporium sphaerospermum*	-	-	-	-
16	*Cladosporium* sp.	-	-	-	-
17	*Colletotrichum guajavae*	-	-	-	-
18	*Colletotrichum lindemuthianum*	-	-	-	-
19	*Colletotrichum gloeosporioides*	++	+	-	-
20	*Colletotrichum tropicale*	-	-	-	-
21	*Colletotrichum* sp.	-	-	-	-
22	*Curvularia* sp.	-	-	-	-
23	*Diplodina* sp.	-	-	-	-
24	*Cylindrocarpon* sp.	-	-	-	-
25	*Fusarium moniliforme*	-	-	-	-
26	*Fusarium oxysporum*	++	+	-	-
27	*Helminthosporium dematioidae*	-	-	-	-
28	*Myrothecium* sp.	-	-	-	-
29	*Nigrospora* sp.	-	-	-	-
30	*Paecilomyces lilacinus*	+++	+++	+++	+++
31	*Penicillium* sp.1	++	+	-	+
32	*Penicillium* sp.2	-	-	-	-
33	*Phoma lingam*	-	-	-	-
34	*Phomopsis archeri*				
35	*Pseudotorula* sp.	-	-	-	-
36	*Pestialopsis* sp.	-	-	-	-
37	*Pythiopsis* sp.	-	-	-	-
38	*Trichocladium* sp.	-	-	-	-
39	*Trichoderma harzianum*	-	-	-	-
40	*Trichoderma* sp.	+++	+++	+++	-
41	*Verticillium* sp.	++	++	-	+
42	*Xylaria* sp.	-	-	-	-
43	Sterile mycelium (hyaline)	-	-	-	-
44	Sterile mycelium (narrow width)	++	+	-	+

Fs-*Fusarium solani*; Fv-*Fusarium verticillioides*; Af-*Aspergillus flavus*; Ao-*Aspergillus ochraceus*

–: no inhibition percent (No activity), **+:** < 20% inhibition (Low activity), **++:** 20-< 50% inhibition (Moderate activity), **+++:** 50-< 70% inhibition (High activity)

Disc diffusion method

Ethyl acetate extract of 20 endophytic fungi that were subjected to disc diffusion assay showed inhibitory activity against at least one of the test pathogens as shown in table 3. *Bipolaris spicifera*, *Colletotrichum gloeosporioides, Colletotrichum tropicale* and *Fusarium verticillioides* showed antibacterial activity against all the 8 test bacteria. The endophytic fungi *Colletotrichum gloeosporioides* showed significant activity with a zone of inhibition (mm±SEM) against all the tested bacteria viz**.** *Xanthomonas campestris* pv. *vesicatora* (36.0±0.56), *Xanthomonas oryzae* (22.3±0.67), *Pseudomonas syringae* (27.6±0.33), *Ralstonia solanacearum* (29.6±0.33), *Bacillus subtilis* (23.6±0.88), *Bacillus cereus* (22.5±0.57), *Escherichia coli* (24.6±0.33) and *Staphylococcus aureus* (23.8±0.88). *Cladosporium oxysporum*, *Fusarium moniliforme*, *Penicillium* sp. and *Phomopsis archeri* showed moderate antibacterial activity against two or more test bacteria.

Paecilomyces lilacinus, Phomopsis archeri and *Trichoderma* sp. showed antifungal activity against all the 4 test fungi. Endophytic fungi *Paecilomyces lilacinus* showed significant antifungal activity against *Fusarium solani* (34.6±0.33), *Fusarium moniliforme* (30.3±0.57), *Aspergillus flavus* (22.3±0.33) and *Aspergillus ochraceus* (23.3±0.88) whereas *Colletotrichum gloeosporioides* showed moderate antifungal activity against *Fusarium solani* (12.3±0.57) and *Fusarium verticillioides* (19.3±0.33). *Phomopsis archeri, Trichoderma* sp., *Penicillium* sp. and *Verticillium* sp. showed moderate activity against one or more test fungi. Negative control did not show any activity against test pathogens.

Table 3: Antimicrobial activity of ethyl acetate extract of fungal endophytes isolated from *P. juliflora* against test pathogens by disc diffusion method

Endophytic fungi	Test pathogens											
	Zone of Inhibition (mm±SE)*											
	Xv	Xo	Rs	Ps	Bs	Bc	Ec	Sa	Af	Ao	Fs	Fv
Alternaria alternate	-	10.6±0.67^{a}	-	13.3±0.88^{bc}	-	-	-	11.3±0.57^{ab}	-	-	-	-
Alternaria sp.	10.5±0.57^{ab}	10.0±0.57^{a}	-	14.6±0.33^{bc}	-	14.6±0.33^{bc}	-	-	NT	NT	NT	NT
Aspergillus terreus	NT	NT	NT	NT	NT	NT	NT	NT	-	-	12.0±0.57^{bc}	9.0±0.57^{a}
Bipolaris spicifera	19.5±0.57^{d}	23.3±0.33^{d}	22.6±0.66^{d}	23.6±0.67^{d}	24.6±0.88	24.3±0.67^{de}	22.3±0.33^{d}	15.6±0.66^{c}	NT	NT	NT	NT
Botryderma	-	24.4±0.33	-	-	-	-	-	-	NT	NT	NT	NT
Botrytis sp.	NT	NT	NT	NT	NT	NT	NT	NT	-	12.3±0.88^{bc}	-	-
Cladosporium sp.	27.0±0.57^{de}	23.3±0.88^{d}	-	-	23.6±0.66	-	-	38.0±0.57^{g}	NT	NT	NT	NT
Cladosporium oxysporum	15.3±0.88^{cd}	33.5±0.57^{fg}	-	-	14.6±0.33	-	24.3±0.88^{de}	11.3±0.88^{ab}	NT	NT	NT	NT
Colletotrichum gloeosporioides	36.0±0.56^{fg}	22.3±0.67^{d}	29.6±0.33^{e}	27.6±0.33^{e}	23.6±0.88^{d}	22.5±0.57^{d}	24.6±0.33^{de}	23.8±0.88^{d}	-	-	9.0±0.57^{a}	19.3±0.33^{bc}
Colletotrichum guajava	25.0±0.57^{de}	15.6±0.33^{c}	-	-	-	-	11.6±0.66^{ab}	-	NT	NT	NT	NT
Colletotrichum tropicale	29.6±0.67^{e}	21.5±0.57^{d}	24.0±0.33^{d}	22.4±0.33^{d}	25.0±0.57	29.3±0.33^{e}	24.3±0.88^{de}	23.4±0.33^{d}	NT	NT	NT	NT
Fusarium oxysporum	NT	NT	NT	NT	NT	NT	NT	NT	-	-	11.3±0.66^{ab}	9.00±0.57^{a}
Fusarium moniliforme	22.6±0.67^{d}	20.0±0.57^{d}	24.0±0.57^{de}	23.3±0.88^{d}	25.3±0.88^{de}	24.3±0.57^{e}	20.0±0.57^{d}	22.0±1.0^{d}	NT	NT	NT	NT
Paecilomyces lilacinus	-	-	19.0±0.57^{d}	19.3±0.33^{e}	-	-	16.0±0.57^{d}	-	22.3±0.33^{d}	23.3±0.88^{d}	34.6±0.33^{fg}	30.0±0.57^{f}
Phomopsis archeri	30.6±0.67^{f}	36.6±0.67^{fg}	-	-	21.3±0.88^{d}	-	-	-	10.0±0.57^{ab}	13.6±0.66^{bc}	15.3±0.57^{d}	11.3±0.57^{ab}
Penicillium sp.1	23.6±0.33^{d}	22.4±0.5^{d}	11.3±0.66^{ab}	15.3±0.33^{c}	-	-	-	21.3±0.88^{d}	12.0±0.57^{bc}	14.3±0.33^{bc}	-	-
Trichoderma sp.1	11.5±0.57^{ab}	-	-	13.8±0.33^{bc}		18.3±0.66^{d}	-	-	15.6±0.33^{c}	11.0±0.57^{ab}	9.3±0.33^{a}	9.3±0.88^{a}
Verticilium sp.	10.0±0.57^{a}	14.6±0.66^{bc}	9.3±0.57^{a}	9.3±0.57^{a}	12.3±0.88^{bc}	-	15.3±0.33^{c}	-	9.0±0.57^{a}	8.3±0.33^{a}	-	-
Sterile mycelium (Hyaline)	-	-	-	17.6±0.33^{de}	11.3±0.88^{ab}	-	-	-	NT	NT	NT	NT
Sterile mycelium (Narrow width)	NT	NT	NT	NT	NT	NT	NT	NT	-	12.0±0.57^{bc}	13.3±0.33^{bc}	-

*Values represent the mean±SE of three replicates. Xv-*Xanthomonas vesicatoria*; Xo-*Xanthomonas oryzae*; Ps-*Pseudomonas syringae*; Rs-*Ralstonia solanacearum*; Bc-*Bacillus cereus*; Bs-*Bacillus subtilis*; Ec-*Escherichia coli*; Sa-*Staphylococcus aureus*; Af-*Aspergillus flavus*; Ao-*Aspergillus ochraceus*; Fs-*Fusarium solani*; Fv-*Fusarium verticillioides*; (-): No Zone of inhibition, (NT): Not Tested.

^{a,b,c,d,e,f,g}Statistical analysis of the data was performed with SPSS 20.0 using Student-Newman-Keuls test for determining significant difference (p = 0.05). ^{a,b,c}low activity, ^{d,e}moderate activity, ^{f,g}high activity.

Minimal inhibitory concentration

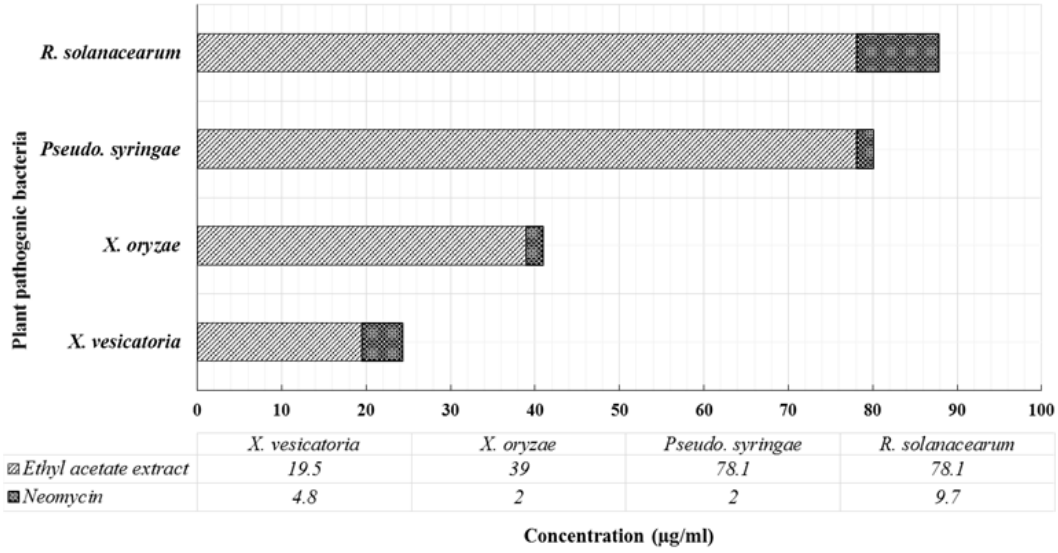
The minimal inhibitory concentrations of ethyl acetate extract of *Colletotrichum gloeosporioides* against the plant and human pathogenic bacteria are presented in fig. 1 and fig. 2 respectively. The MIC varied between 5000 µg/ml and 2 µg/ml. The ethyl acetate extract of *Colletotrichum gloeosporioides* showed lower MIC against *Xanthomonas vesicatoria* and *Xanthomonas oryzae* with concentrations of 19 µg/ml and 39 µg/ml, respectively, whereas the positive control Neomycin recorded the lowest MIC value of 2 µg/ml against *Xanthomonas oryzae* and *Pseudomonas syringae*.

The MIC of ethyl acetate extract of *Colletotrichum gloeosporioides* was low (78.1 µg/ml) for *E. coli* and was highest (312 µg/ml) for *Bacillus cereus*. Gentamycin recorded the MIC of 4.8 µg/ml against *E. coli* and *staphylococcus aureus* as shown in fig. 2.

The MIC of ethyl acetate extract of *Paecilomyces lilacinus* and fungicide Carbendazim against plant pathogenic fungi are presented in fig. 3. *Paecilomyces lilacinus* ethyl acetate extract showed lowest MIC against *Fusarium solani* (9.7 µg/ml) and the highest MIC was against *Aspergillus flavus* and *Aspergillus ochraceus* (156.2 µg/ml). The systemic fungicide Carbendazim showed the lowest MIC value of 4.8 µg/ml against *Fusarium verticillioides* and *Fusarium solani*.

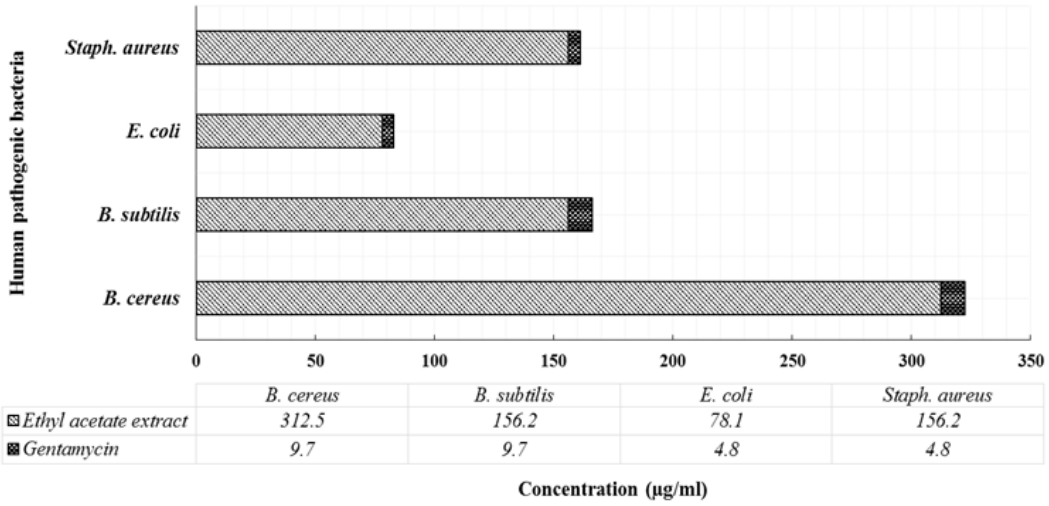
Biochemical screening

The ethyl acetate extract of *Colletotrichum gloeosporioides* and *Paecilomyces lilacinus* showed the presence of alkaloids, carbohydrates, sterols and coumarins. Flavonoids were found to be present in *Colletotrichum gloeosporioides* but absent in *Paecilomyces lilacinus* as shown in table 4.



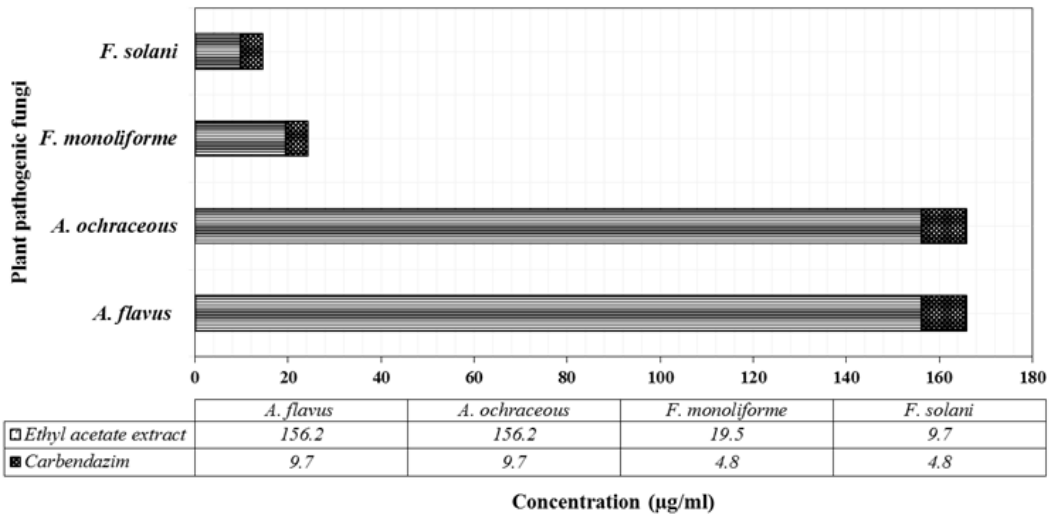
	X. vesicatoria	X. oryzae	Pseudo. syringae	R. solanacearum
Ethyl acetate extract	19.5	39	78.1	78.1
Neomycin	4.8	2	2	9.7

Fig. 1: Minimal inhibitory concentration of ethyl acetate extract of *Colletotrichum gloeosporioides* against plant pathogenic bacteria



	B. cereus	B. subtilis	E. coli	Staph. aureus
Ethyl acetate extract	312.5	156.2	78.1	156.2
Gentamycin	9.7	9.7	4.8	4.8

Fig. 2: Minimal inhibitory concentration of ethyl acetate extract of *Colletotrichum gloeosporioides* against human pathogenic bacteria



	A. flavus	A. ochraceous	F. monoliforme	F. solani
Ethyl acetate extract	156.2	156.2	19.5	9.7
Carbendazim	9.7	9.7	4.8	4.8

Fig. 3: Minimal inhibitory concentration of ethyl acetate extracts of *Paecilomyces lilacinus* against plant pathogenic fungi

Table 4: Biochemical characterization of ethyl acetate extract of *Colletotrichum gloeosporioides* and *Paecilomyces lilacinus*

S. No.	Biochemical test	Endophytic fungi	
		Colletotrichum gloeosporioides	*Paecilomyces lilacinus*
1.	Alkaloids		
a.	Mayer's test	+	+
b.	Wagner's test	+	+
2.	Carbohydrates		
a.	Molisch's test	+	+
b.	Fehling's test	+	+
3.	Proteins & amino acids		
a.	Ninhydrin test	-	+
b.	Biuret test	-	+
4.	Steroids		
a.	Liebermann Burchard's test	-	-
5.	Saponins		
a.	Foam test	-	-
6.	Flavonoids		
a.	Alkaline Reagent test	-	-
b.	H_2SO_4 test	+	-
c.	Shinoda test	+	+
7.	Tannins		
a.	Ferric Chloride test	-	-
8.	Quinones		
a.	Conc. HCl test	-	-
9.	Sterols		
a.	Salkowski's test	+	+
b.	Liebermann Burchard's test	+	+
10.	Phenols		
a.	Ferric chloride test	-	-
11.	Anthocyanin		
a.	NaOH test	-	-
12.	Phlobatanins		
a.	HCl test	-	-
13.	Cardiac glycosides		
a.	Keller killani test	-	-
14.	Anthraquinones		
a.	Borntrager test	-	-
15.	Carotenoids		
a.	H_2SO_4 test	-	-
16.	Coumarins		
a.	Fluorescence test	+	+

+: Present,-: Absent

DISCUSSION

Over the last few decades, endophytic microorganisms have gained immense importance as a valuable natural resource for imminent utilization in diverse areas such as agriculture and biotechnology [3, 37]. Currently, there is a pressing need for the search of new antimicrobial agents because of the development of resistance in pathogens to many available drugs. A number of bioprospecting strategies are engaged in order to discover competent endophytes with desirable traits. For instance, endophytes could be isolated from randomly sampled plants from different population, or initially performing a detail investigation of an ecosystem in order to determine its feature with regard to its natural population of plant species, their relationship with the environment, soil composition and biogeochemical cycles, followed by endophyte isolation and characterization [4, 37].

Thus, in order to screen for the most promising endophytes, we estimated the potential of the isolated endophytic fungi as bio-control agents by challenging them with pathogenic bacteria and fungi and as a substitute for existing drugs by checking the antimicrobial potential toward plant and human pathogenic microbes. The knowledge of the chemistry and biology of endophytes would pave a way for the isolation and characterization of antibacterial and antifungal bioactive principles that may help to replace the existing synthetic drugs to overcome the resistance of pathogens and may also help us to manage plant pathogens without contaminating the environments. Herein we report for the first time, the bioprospecting of fungal endophytes isolated from leaves of *P. Juliflora*. The rationale used here is that *P. Juliflora* contains a number of antimicrobial compounds and might also harbour competent endophytes capable of providing fitness benefits to the host plant. Such benefit could encompass the endophytes producing a plethora of bioactive compounds, even the ones exclusive to the associated plant, thereby assisting in the chemical defense of the host against invading pathogens [4, 6, 8, 25].

In the present study, Hyphomycetes, a class of Deuteromycotina, ranked first followed by Coelomycetes, *Mycelia sterilia* and Ascomycetes. Hyphomycetic fungi are common endophytes among plants inhabiting in temperate, tropical and rainforest vegetation. Findings of the present study are in conformity with previous studies, [11] in which member of the subdivision Deuteromycotina is most abundant. In our study, species of *Alternaria*, *Cladosporium*, *Colletotrichum*, *Fusarium, Penicillium* and *Trichoderma* are the frequently isolated genera. Interestingly, these genera dominated over the most cosmopolitan species of *Aspergilli* which suggest that it may be due to substrate specificity. These results agree with other reports on endophytes where it is reported that many fungal species can be isolated from a given host, but a few are frequently found [2, 8, 24].

In the literature, the genera *Alternaria*, *Colletotrichum*, *Phomopsis* and *Cladosporium* are reported to be found most often as endophytes in diverse host plant tissues [18-26]. In addition to the core group of species frequently isolated, our study also reported the occurrence of a few incidental species such as *Aspergillus terreus*, *Botrytis* sp., *Botryderma* sp., *Pseudotorula* sp. and *Pythiopsis* sp.

which were isolated only in a small number of samples. Each incidental species are usually found only once or twice in several hundred samples [6]. In general, the number of rare or incidental species isolated is proportionate to the intensity of sampling [2]. Our results indicate that sampling was quite adequate to discover even rarer species.

All the isolated fungal endophytes from *P. Juliflora* were subjected to antibacterial and antifungal assay on solid medium. In total, 17 out of 44 endophytic fungi showed antibacterial activity against the plant and human pathogenic bacteria and 10 endophytic fungi showed antifungal activity against plant pathogenic fungi. Our results are consistent with earlier reports on other plants and among strains of endophytes more than 30% had antibacterial and antifungal activities [6, 11]. Ethyl acetate extract of twenty fungal isolates (45.5%) showed a broad spectrum of antimicrobial activity and inhibited Gram-positive, Gram-negative bacteria and plant pathogenic fungi.

Among all tested endophytic fungi, *Colletotrichum gloeosporioides* showed significant antibacterial activity against one or more test pathogens whereas *Alternaria alternata, Bipolaris* sp., *Cladosporium oxysporum, Colletotrichum tropicale* and *Fusarium verticillioides* showed moderate antibacterial activity against both plant and human pathogenic bacteria. The inhibition results of ethyl acetate extract differ from the results of dual culture as many of the ethyl acetate extracts of endophytic fungi like *Alternaria alternata, Aspergillus terreus* and sterile mycelia did not show antibacterial activity. As secondary metabolite production requires specific cultural conditions such as temperature, pH and humidity for the growth of fungi [2]. In other studies of medicinal plants, *Alternaria alternata*, *Alternaria* sp., *Colletotrichum* sp. and *Phomopsis* sp. have been isolated and found to show activity against the bacteria *Staphylococcus aureus*, *S. aureus* methicillin-resistant, *Escherichia coli* and *Bacillus subtilis* [23-26]. An antibacterial naphthoquinone like javanicin was reported from an endophyte *Chloridium* sp. isolated from neem [22]. Antimicrobial volatile organic compounds (VOCs) were reported from the mitosporic xylariales fungi *Muscodor albus* and *M. vitigenus* isolated from *Cinamomum zeylanicum* [6].

Among all tested endophytic fungi, ethyl acetate extract of *Colletotrichum gloeosporioides* was tested for MIC against the plant and human pathogenic bacteria and further biochemical screening were done to identify broadly the antibacterial bioactive principles. Ethyl acetate extract of *Colletotrichum gloeosporioides* showed MIC in the range of 19.7-39.8 µg/ml which was compared with standard antibiotics. The biochemical screening of *Colletotrichum gloeosporioides* showed the presence of major groups of secondary metabolites such as alkaloids, sterol, flavonoids and coumarins**.** Earlier researchers have also isolated antimicrobial compounds from *Colletotrichum* species in general and *Colletotrichum gloeosporioides* in particular [39, 25, 26]. The characterization of antimicrobial metabolites from the culture of *Colletotrichum* species shows that the endophytic fungus is presumably involved in the superior adaptability and competitiveness in nature [18].

Endophyte *Paecilomyces lilacinus* showed significant antifungal activity against plant pathogenic fungi. Other endophytic fungi like *Penicillium* sp., *Trichoderma* sp. and *Verticillium* sp. recorded moderate antifungal activity against one or more plant pathogenic fungi. Several potent antifungal compounds have been identified and characterized from endophytic fungi *Chaetomium* sp., *Alternaria* sp., *Botryodiplodia* sp., *Cladosporium* sp., *Fusarium* sp., *Penicillium* sp., *Pestialopsis* sp., *Phomopsis* sp. and *Trichothecium* sp. isolated from different hosts [19, 21, 38].

Paecilomyces lilacinus showed significant antifungal activity against plant pathogenic fungi. The minimum inhibitory concentration was in the range of 19.7-39.8 µg/ml against the phytopathogens. The biochemical screening also showed the presence of major groups of secondary metabolites such as alkaloids, carbohydrates, sterol, flavonoids and coumarins. *Paecilomyces lilacinus* is a known biopesticide against nematodes of root knot of mulberry. It produces a serine protease that is toxic to *Meloidogyne* sp. eggs [40]. A novel nematicidal compound 4-(4'-carboxy-2'-ethyl-hydroxypentyl)-5,6-dihydro-6methylcyclobuta[b]pyridine-3,6dicarboxylic acid has been isolated from *Paecilomyces lilacinus* that weaken the nematodes and then gets inside the insect and kills the infected nematode [41]. Present work is the first report of the antifungal potential of the endophyte *Paecilomyces lilacinus* against plant pathogenic fungi.

Thus, the antimicrobial potential of fungal endophytes also accounts for the medicinal properties of *P. Juliflora*. Further purification of the bioactive principle will result in more significant activity. These secondary metabolites constitute diverse structures and are responsible for many biological activities. Recent reports show hundreds of natural products including alkaloids, flavonoids, and steroids obtained from endophytes. The bioactive compounds isolated from these endophytes are known to have antibiotics, immunosuppressant, anticancer agents and biological control agents [7, 21, 24-26]. The preliminary screening has confirmed the antimicrobial potential of the fungal endophytes of *P. Juliflora*.

CONCLUSION

The results of present study reveal that endophytic fungi harbored in leaves of *P. Juliflora* have great promise, not only as biocontrol agents against the known and emerging phytopathogens and human pathogenic bacteria but also as a sustainable resource of biologically active novel secondary metabolites, which can be developed as an herbal drug. Further, the finding of this study provides a strong platform for the isolation and purification of novel natural antimicrobial agents from endophytic fungi of *P. Juliflora* using analytical chemistry.

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CONFLICTS OF INTERESTS

All authors have none to declare.

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