

Endophytic mycobiota recorded from *Clerodendrum inerme* and their biological activities

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ABSTRACT

Endophytic fungi were isolated from the medicinal plant, *Clerodendrum inerme* (L.) Gaertn., which was collected from Western Ghats of Sathyamangalam Tiger Reserve, Sathyamangalam, Erode District, Tamil Nadu. As many as 150 segments were screened from leaves, twigs and shoots of selected medicinal plant. A total of 31 species which belong to 31 genera were recorded among which 12 isolates were classified into sterile morpho species. Biodiversity indices such as Gleason index (G) and Shannon index of leaf, shoot, twigs shows the maximum species diversity. Jaccard's similarity coefficient represents the maximum similarity in leaves-twigs (0.20) followed by leaves-shoot (0.2631) and twigs-leaves (0.1764). The selected endophytic fungal strains were mass cultured in modified PDB medium at 27±2°C for 14 days. Mycelia and broth were separated by filtration. The mycelial cells were ultra sonicated and the biological compounds were extracted through chloroform and methanol. The compounds were tested and also quantified, the antioxidant property was evaluated by employing DPPH, FRAP, FTC, TPC, and TBA assays. The antioxidant and free radical scavenging activity recorded was around 99% inhibition at the concentration of 6.25-20mg/mL. The total phenolic content of 3.142-4.445±2.85 µg/mL was evaluated and compared with standard Tannic acid. The endophytic fungal extract was evaluated for its antibacterial activities. Agar well diffusion exhibits the inhibition range of 15-22mm. The Minimum Inhibitory Concentration showed the antibacterial potency at lower concentration of 12.5-25µg/mL with all gram positive and gram negative bacterial strains studied.

KEYWORDS: Endophytes, Biodiversity indices, Minimum inhibitory concentration, DPPH, TPC.

INTRODUCTION

Endophytes are microorganisms that are bacteria or fungi living in plant tissues without causing negative effects to host and capable of producing the natural products useful in medicine and agriculture based industry (Kumar and Sagar, 2007; Bacon and White, 2000; Strobel and Daisy, 2009). Certain tropical forest regions of Guyana and Mudumalai revealed the hyper diverse nature of fungal endophytes. (Cannon and Simmons, 2002; Suryanarayanan *et al.*, 2002). Endophytes of *Curvularia* sp. Isolated from petiole, bark and leaf of *Alstonia scholaris* L. showed antibacterial activity (Mahapatra and Banerjee, 2010). Endophytes are known to produce rich source of natural products to overcome the pathogenic invasion (Tan and Zou, 2001).

It is a common belief that the photochemical and bioactive products produced by endophytes are indicative of the host specificity relationship (Wani *et al.*, 1971). These are reported to have a role in the production of volatile compounds (Bicas *et al.*, 2009; Krings *et al.*, 2006). Metabolites having antibacterial activity are reported to be useful in pharmaceutical industries (Guo *et al.*, 2008). The antimicrobial agents are not only documented to be useful as antibiotics but also in food industry for preservation (Liu *et al.*, 2008). Coumarin, an important bioactive compound was isolated from endophytic fungus *Pestalotiopsis versicolor* (Speg.) Steyaert, along with other known compounds (Yang *et al.*, 2015). *Mucor irregularis* Stichigel, Guarro & Ed. Alvarez, known to be associated with the medicinal plant *Moringa stenopetal* (Baker f.) Cufod. found in Cameroon, is reported to show biological activity (Akone *et al.*, 2016). Endophytes are also reported to be an important source of novel sesquiterpenes and some of the already known terpenes (Liu *et al.*, 2015).

Natural antioxidants are reported to be of common occurrence in the medicinal plants, vegetables and fruits (Liu *et al.*, 2007; Yu *et al.*, 2010; Huang *et al.*, 2017). Endophytic microorganisms are known to produce polysaccharides with

antioxidant properties (Liu *et al.*, 2009). Some of the well known endophytes are reported to produce volatile organic compounds such as cycloalkenes, cycloalkanes and benzene derivatives (Malette, *et al.*, 2014). The primary objective of the present study is to isolate and identify the endophytic fungi from *Clerodendrum inerme* (L.) Gaertn., and to study their antimicrobial and antioxidant activity.

MATERIALS AND METHODS

Plant sample collection: The medicinal plant of *Clerodendrum inerme* belongs to family *Lamiaceae*. It is commonly known as 'Sanganguppi' in Tamil dialect. The plant sample was taken from Thalamalai range of Sathyamangalam Tiger Reserve (STR) and kept in paper bags and processed within 24 hours following collection (Petrini, 1986).

Isolation and Identification of endophytic fungi: The endophytic fungi were isolated from *Clerodendrum inerme* through modified surface sterilization technique. These were subsequently identified based on morphological and microscopic features consulting authentic literature (Guba 1961; Ellis 1971; Sutton 1980 and Nag Raj 1993). The identified endophytes were cry preserved at Endophytic Fungal Culture Collection Centre (EFCCC), Department of Biotechnology, BIT, and Sathyamangalam.

Statistical Analysis: Relative Percentage Occurrence, Colonization Frequency, Jaccard's Similarity Co-efficient and species diversity (Shannon Diversity index and Gleason index) were calculated for the endophytic assemblage (Suryanarayanan *et al.*, 2002; Kannan, 2017).

Selection of medium and endophytic fungal positive strain: The shake flask experiment was carried out for preliminary screening of secondary metabolites. The pre-dominant endophytic fungi were cultured in 100 mL of modified Potato Dextrose Broth for undertaking studies pertaining to antioxidant activities. For the screening of antimicrobial compounds culturing was done in 250 mL Erlenmeyer flasks followed by

incubation at 27±2°C for 14 days.

Extraction of cell-free extract for screening of secondary metabolites: Mycelia and broth were centrifuged at 10,000 rpm for 10 minutes at 4°C. The pellet was transferred into a sterile container and dried overnight in a hot air oven at 40°C. The dried mycelia and methanol was pulverized with the help of ultra sonicator at 100% frequency and the extraction was repeated with chloroform and methanol mixed in the ratio of 4:1. The crude fungal compounds were extracted and further subjected to preliminary screening for secondary metabolite. The crude was stored at 4°C for further studies (Mussagy *et al.*, 2019; Zhang *et al.*, 2018).

Procurement of Test Bacteria: Six bacterial strains, namely *Bacillus subtilis* (MTCC 121), *Escherichia coli* (MTCC 433), *Staphylococcus aureus* (MTCC -3160), *Klebsiella plenticola* (MTCC - 530), *Proteus vulgaris* (MTCC 426), and *Lactobacillus lactis* (MTCC 4185) were procured from Microbial Type Culture Collection (MTCC), Gene Bank, Chandigarh, India. The stock cultures were maintained on slants of Nutrient agar at 4°C. Active cultures for screening their susceptibility were prepared by transferring loop full of cultures from stock cultures to test tubes containing nutrient broth which were incubated at 37°C for 24 hours using Micro broth dilution method. The fungal extracts were screened against 4 bacterial strains. (Tamilarasan *et al.*, 2013).

Determination of Minimum Inhibitory Concentration (MIC): The fungal extract (100 µg/mL) as an initial concentration was taken in the first well of 96 well titre plate. This was serially diluted resulting in 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL, 1.6 µg/mL and finally 0.8 µg/mL of extract in series. Each well with respective concentration of fungal extract was inoculated with 0.01 mL of 24 hours bacterial cell suspension. Streptomycin was used as a positive control and methanol was used as a negative control. This was incubated at 37°C for 24 hours. The presence of turbidity of the broth indicates positive growth (Udayaprakash *et al.*, 2013).

DPPH Radical Scavenging Assay: Different reactions of methanolic extracts of fungal sources (10-50 mg/mL) were taken and the total volume was made up to 250 µL with chloroform and methanol, respectively. 1 mL of DPPH (4 mg/100 mL) was added and the tubes were kept in dark for incubation at room temperature for 20 min. The absorbance was checked against the blank at 517 nm. Per cent free radical scavenging was calculated based on the extent of reduction in the colour (Udayaprakash *et al.*, 2015).

The per cent radical scavenging activity was calculated by applying the following relation:

$$\% \text{ Radical scavenging} = \left\{ \frac{Ac - As}{Ac} \right\} \times 100$$

Where Ac = Absorbance of control and As = Absorbance of test sample.

Ferric Reducing Antioxidant Power (FRAP) Assay : 1 mL of fungal extract, 2.5 mL phosphate buffer (of 0.2 M, pH 7) and 1% potassium ferri cyanide (2.5 mL) were mixed and incubated at 50°C for 30 min. To this solution, 2.5 mL of 10%

trichloro acetic acid was added and centrifuged at 6500 rpm for 10 min. Distilled water (2.5 mL) and then 0.5 mL of 0.1% Ferric chloride were added to 2.5 mL of the supernatant. The absorbance of the solution was measured at 700 nm using UV-visible spectrophotometer. The reducing ability of the fungal extract was evaluated in terms of percentage by relating to the standard, Ferrous sulphate (Udayaprakash, *et al.*, 2015).

$$\text{Percentage inhibition} = \frac{\text{Absorbance of the control (Ac)} - \text{Absorbance of the sample (As)}}{\text{Absorbance of the control (Ac)}} \times 100$$

Ferric Thiocyanate (FTC) Assay: FTC assay involves the addition of 120 µL of 98% ethanol, 100 µL of 2.51% linoleic acid in ethanol and 9 mL of 40 mM phosphate buffer (pH 7) to 100 µL of the fungal extract. To 100 µL of the mixture, 9.7 mL of 75% ethanol, 100 µL of 30% ammonium thiocyanate and 100 µL of 20 mM FeCl₃ in 3.5% HCl were added after maintaining the solution in the dark, at 40°C. The absorbance of the solution was measured at 500 nm using UV-visible spectrophotometer after 3 min. The percentage of inhibition was calculated with Tannic acid as the standard (Udayaprakash *et al.*, 2015).

$$\% \text{ Inhibition} = \frac{\text{Absorbance of the control (Ac)} - \text{Absorbance of the sample (As)}}{\text{Absorbance of the control (Ac)}} \times 100$$

Thiobarbituric Acid (TBA) Assay: Equal volume (200 µL) of 20% trichloro acetic acid and 0.67% thio-barbituric acid were mixed with 1 mL of 2.51% linoleic acid and 1 mL of fungal extract. The solution was maintained in boiling water bath for 10 min; cooled to room temperature and centrifuged at 3000 rpm. The percentage inhibition of the fungal sources against the secondary metabolites of lipid peroxidation was evaluated with reference to the standard solution of butylated hydroxyl toluene (BHT). The supernatant was subjected to UV-visible spectrophotometric analysis at 532 nm (Udayaprakash *et al.*, 2015).

$$\% \text{ Inhibition} = \frac{\text{Absorbance of the control (Ac)} - \text{Absorbance of the sample (As)}}{\text{Absorbance of the control (Ac)}} \times 100$$

Total Phenolic Content (TPC) Assay: FolinCiocalteu method was followed for the determination of the total phenolic content of the fungal extract. Distilled water (500 µL) and FolinCiocalteu reagent (100 µL) were added to 100 µL of the plant extract and incubated for 6 min at room temperature. The final volume was made up to 3 mL after addition of 1.25 mL of 7% sodium carbonate. The total phenolic content was expressed as milligrams of tannic acid equivalents per gram of dry weight (mg TAE/g DW) of the fungal extract, using a standard plot of Tannic acid. The absorbance was measured at 760 nm using UV-visible spectrophotometer (Cyber-Lab, USA) after an incubation period of 90 min (Barreira *et al.*, 2008).

RESULTS

Isolation and Identification of endophytic fungi and Statistical Analysis: In this present study, in all 150 segments were screened from leaves, twigs, shoot of the selected medicinal plant. From the screened segments the isolated twelve microbes were classified into sterile morpho-species

while eighty identified sporulating species belonged to 31 genera. Out of these *Pestalotiopsis karstenii*, *Phyllosticta* sp., *Colletotrichum* sp., *Phomopsis* sp., *Xylaria* sp., *Alternaria alternata* and *Nigrospora sphaerica*, were found to be dominant endophytic taxa (Figs. 1-2).



Fig. 1: Habitat of *Clerodendrum inerme*

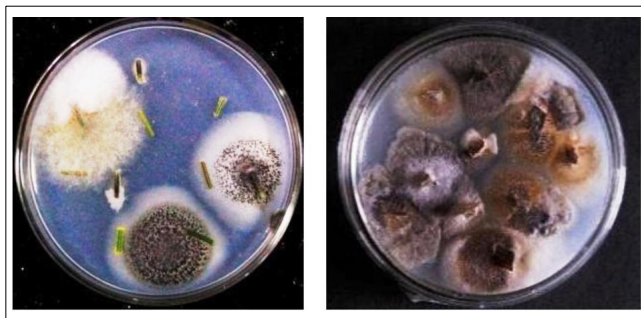


Fig. 2: Endophytic fungal propagules emerging out from tissues of *Clerodendrum inerme*

Colonization Frequency of different plant parts of *Clerodendrum inerme*: A total of 10 endophytic fungal species were recorded from the twig sample, which belonged to 8 genera and 2 sterile forms. In this sample the colonization frequency of endophytic fungal taxa was found to be dominated by coelomycetous fungi followed by hyphomycetous fungi and sterile forms. In shoot sample also a total of 10 endophytic fungal species were recorded, which belonged to 6 genera and 3 sterile forms. In this the colonization frequency of endophytic fungi was again found to be dominated by coelomycetous members followed by hyphomycetous fungi, some members of *Ascomycetes* and sterile forms. In case of leaves a total of 14 endophytic fungal species were recorded, which belonged to 8 genera and 6 sterile forms. Here also the colonization frequency of endophytic fungi was dominated by coelomycetous fungi followed by sterile forms, hyphomycetous fungi and some *Ascomycetes* (Fig: 3).

Relative Percentage Occurrence (RPO) of endophytes in different plant parts: The maximum RPO amongst the different groups of endophytic fungi from the twig sample was of coelomycetous fungi (65%) followed by

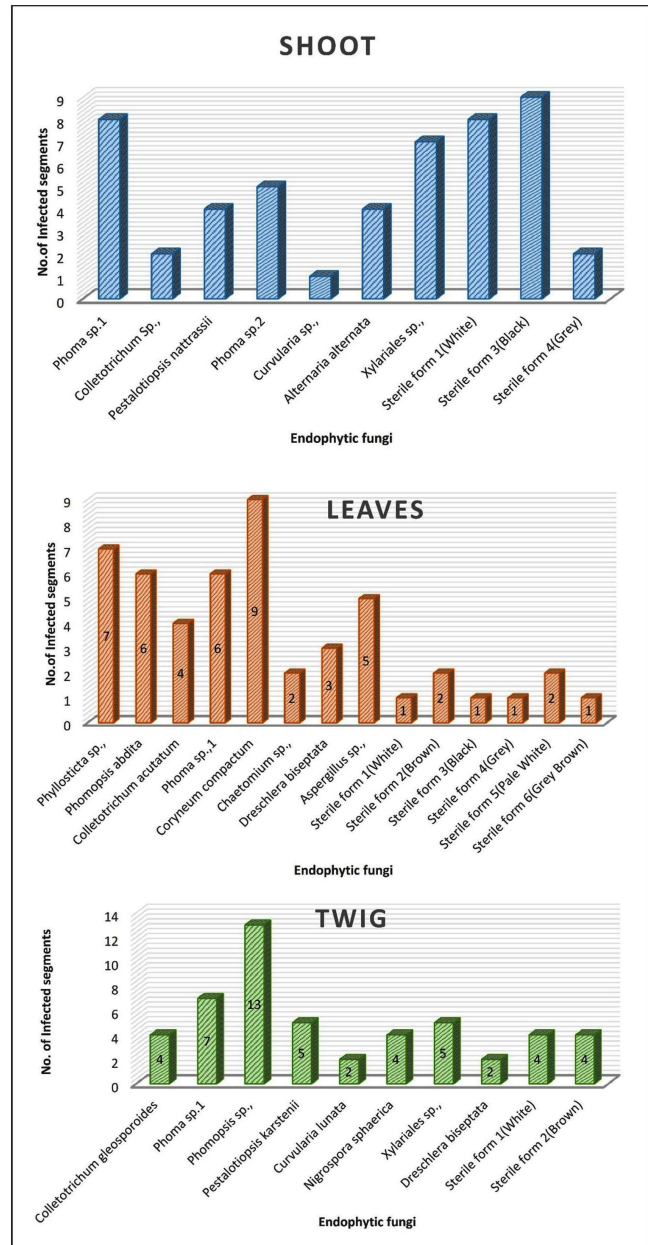


Fig. 3: Colonization Frequency of endophytes recorded from the tissues of leaves, twigs and shoots of *Clerodendrum inerme*.

hyphomycetous fungi (25%) and sterile forms (22%). Similarly in the shoot sample coelomycetous endophytic fungi (64%) were maximum followed by hyphomycetous fungi (9%), *Ascomycetes* (8%) and sterile forms (19%). In the leaves sample as well maximum relative percentage was of endophytic coelomycetous members (38%) followed by hyphomycetous members (32%), *Ascomycetes* (4%) and sterile forms (26%) (Fig. 4).

Jaccard's Similarity Co-efficient: The Jaccard similarity Co-efficient of Leaves and shoot was found to be 26% followed by that of leaves and twigs (20%) and twig and shoot (17%). (Fig. 5)

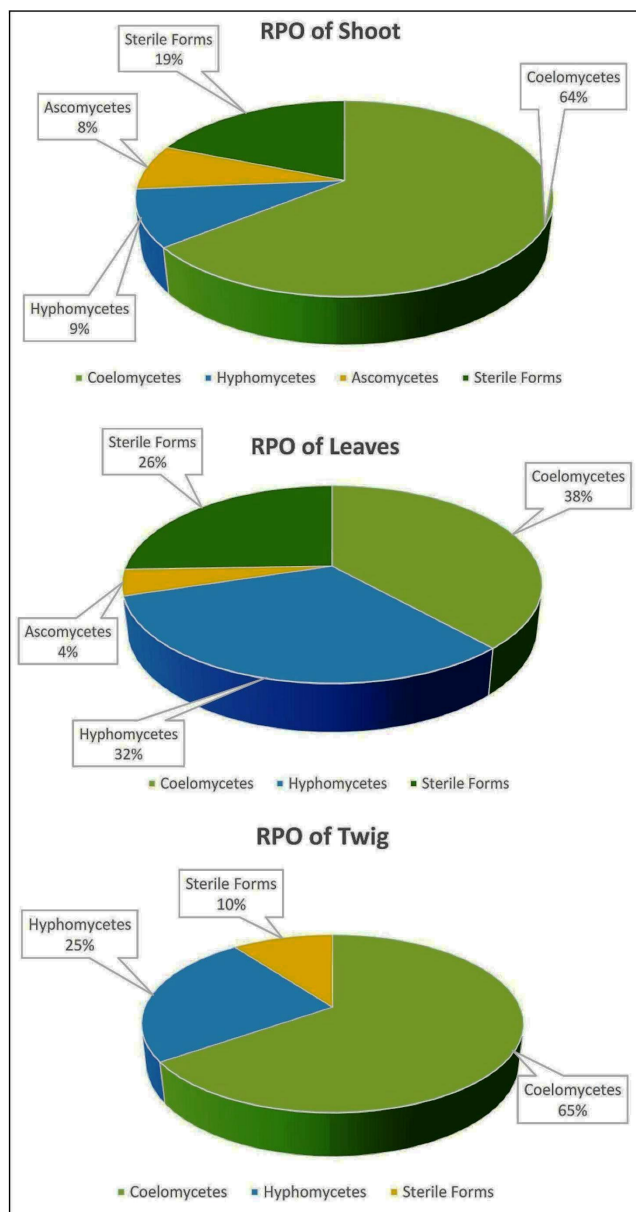


Fig. 4: Relative Percentage occurrence of endophytes recorded from tissues of leaves, twigs and shoots of *Clerodendrum inerme*.

Biodiversity Indices of fungal endophytes in different plant parts: The Biodiversity Indices of fungal endophytes in twig (Gleason Index :2.3125; Relative Index of Gleason: 0.1875; Shannon Diversity Index: 0.25715; Shannon Evenness:0.11168; Relative Index of Shannon: 0.06607), shoot (Gleason Index: 2.3005; Relative Index of Gleason:0.1836; Shannon Diversity Index: 0.2752; Shannon Evenness: 0.1195; Relative Index of Shannon: 0.0703) and the leaves (Gleason Index: 3.3230; Relative Index of Gleason: 0.2653; Shannon Diversity Index: 0.2752; Shannon Evenness : 0.1043; Relative Index of Shannon:0.0703) are depicted in **Fig. 6**.

Agar Well Diffusion Plate Assay for antibacterial activity: The endophytic fungal extract was evaluated for its

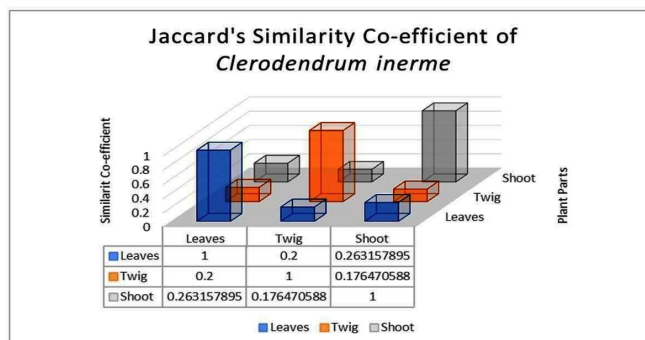


Fig. 5: Jaccard's Similarity Co-efficient of *Clerodendrum inerme*.

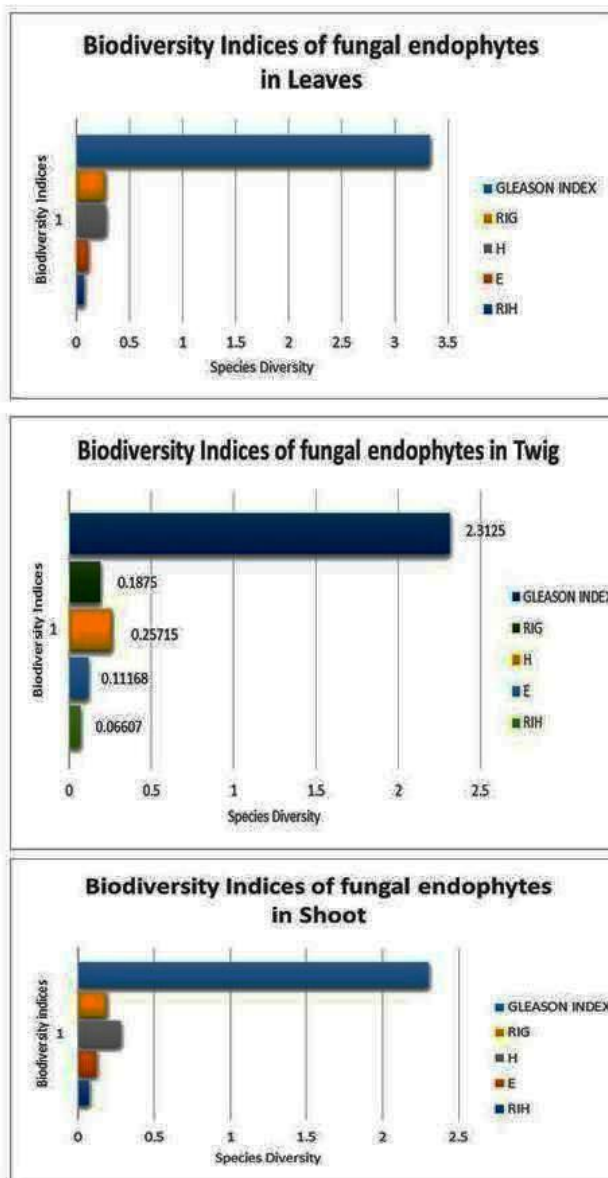


Fig. 6: Biodiversity Indices of Leaves, Twig and Shoots of *Clerodendrum inerme*.

antibacterial activities by agar well diffusion plate assay which exhibited the inhibition range of 15-22 mm (**Table 1**). The minimum inhibitory concentration showed the antibacterial potency at lower concentration (12.5-25 μ g/mL)

Table 1: Antibacterial activities of *Colletotrichum* sp. 1 compared with Positive control through Agar-well Diffusion Method

S. No	Organism	Control Streptomycin Diameter (mm)	Fungal Extract Diameter (mm) <i>Colletotrichum</i> sp. 1
1	<i>Bacillus subtilis</i> (MTCC 121)	20 µL - 22±3.1 40 µL - 29.21±6.34 60 µL - 31.62±2.12	20 µL - 6.3 ±3.50 40 µL - 12±1.34 60 µL - 15.4 ± 1.45
2	<i>Staphylococcus aureus</i> (MTCC 3160)	20 µL - 18.78±1.91 40 µL - 25.41±2.61 60 µL - 29.41±2.18	20 µL - 9.21±3.47 40 µL - 12.86±1.13 60 µL - 21.68±2.12
3	<i>Lactobacillus lactis</i> (MTCC 4185)	20 µL - 20±2.21 40 µL - 26.22±2.38 60 µL - 31.33±1.52	20 µL - 10.11±1.28 40 µL - 15.39±2.35 60 µL - 18.31±1.18
4	<i>Proteus vulgaris</i> (MTCC 426)	20 µL - 17.37±1.8 40 µL - 22.16±1.13 60 µL - 26.33±2.12	20 µL - 11.94±1.03 40 µL - 16.10±1.81 60 µL - 20.13±1.89
5	<i>Escherichia coli</i> (MTCC 433)	20 µL - 19.34±3.17 40 µL - 24.46±1.18 60 µL - 28.35±1.39	20 µL - 11.27±2.14 40 µL - 14.22±2.21 60 µL - 19.27±1.01
6	<i>Klebsiella plenticola</i> (MTCC 530)	20 µL - 14.81±1.02 40 µL - 19.03±0.78 60 µL - 28±1.56	20 µL - 7.29 ±1.47 40 µL - 11.12±1.38 60 µL - 17.23±1.39

with all gram positive and gram negative bacterial strains studied (Table 2).

Quantification of antioxidant assays: The antioxidant and free radical scavenging activities were documented around 99% inhibition at the concentration of 6.25-20 mg/mL. The endophytic fungal extract was evaluated for antioxidant and free radical scavenging ability through DPPH, FRAP, FTC,

Table 2: Minimum Inhibitory Concentration of *Colletotrichum* sp. 1., compared with positive control

	<i>Bacillus subtilis</i> (MTCC 121)	<i>Staphylococcus aureus</i> (MTCC 3160)	<i>Lactobacillus lactis</i> (MTCC 4185)	<i>Proteus vulgaris</i> (MTCC 426)	<i>Klebsiella plenticola</i> (MTCC 530)	<i>Escherichia coli</i> (MTCC 433)
	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml
Fungal Extract	12.5	6.25	12.5	25	6.25	12.5
Positive	3.125	3.125	3.125	3.125	3.125	3.125
Negative	25	25	50	50	50	No Inhibition

TBA and TBA assay (Figs. 7-11). The total phenolic content of was found to be 3.142-4.445±2.85 µg/mL compared with Standard Tannic Acid (Fig. 11).

DISCUSSION AND CONCLUSION

Antioxidant compounds from endophytic fungi are known to have anti-inflammatory, anti-tumor, antibacterial and antiviral activity (Owen, 2002; Sala *et al.*, 2002; Halliwell,

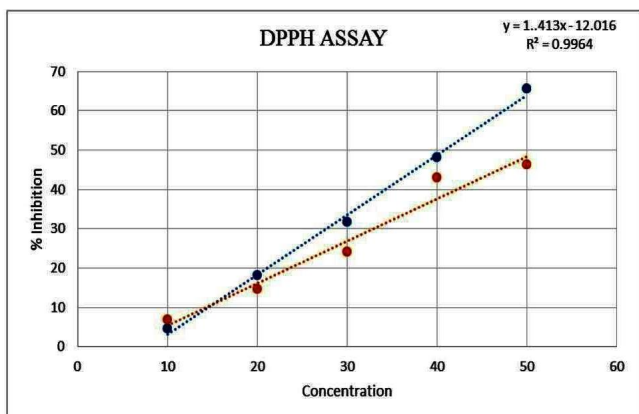


Fig. 7: Quantified DPPH assay of *Colletotrichum* sp. 1

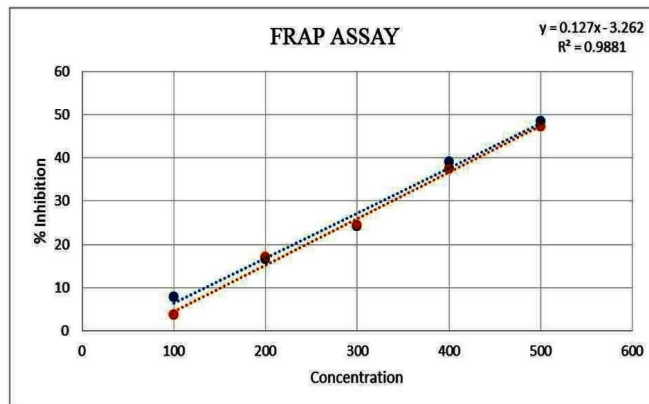


Fig. 8: FRAP quantification of *Colletotrichum* sp. 1

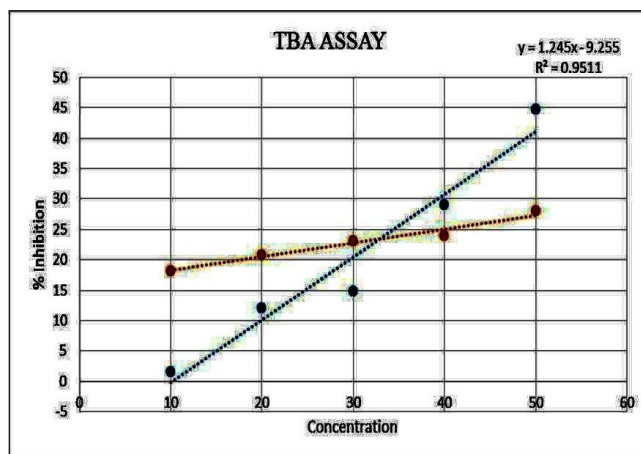


Fig. 9: TBA quantification of *Colletotrichum* sp. 1

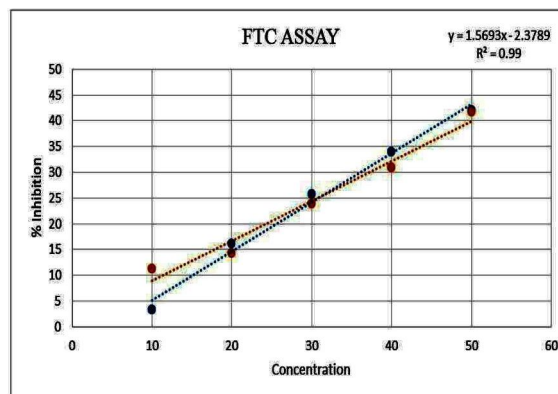


Fig. 10: Quantification of FTC assay for *Colletotrichum* Sp. 1

1994). Endophytic fungi known to produce antibiotic substances are reported to be play an important role in pathogenic conditions (Guo *et al.*, 2008). In this present study, 150 segments were screened from leaves, twigs and shoots of the selected medicinal plant. Out of the total isolated endophytic fungi, twelve isolates were classified into sterile morphospecies and eighty sporulating species belonging to 31 genera. Out of these *Pestalotiopsis karstenii*, *Phyllosticta* sp., *Colletotrichum* sp., *Phomopsis* sp., *Xylaria* sp., *Alternaria alternata*, *Nigrospora sphaerica*. were found to be quite dominant in the host

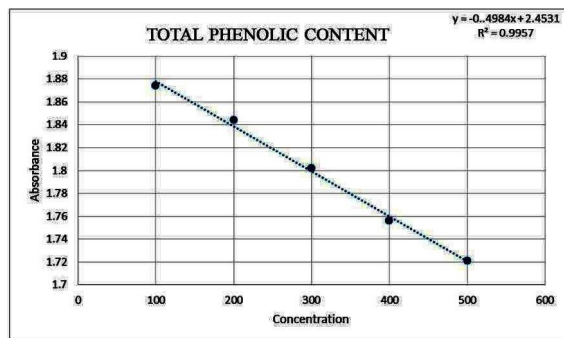


Fig. 11: Quantification of Total Phenolic Content from *Colletotrichum* sp.1

tissues. Dominant endophytic fungi primarily belonged to *Coelomycetes* followed by *Hyphomycetes*, Sterile forms and *Zygomycetes*. Relative percentage occurrence showed that *Coelomycetes* were dominant in shoot, sterile forms in leaves and twig. Jaccard's similarity coefficient was maximum for leaves-twigs (0.20) followed by leaves-shoot (0.2631) and twigs-leaves (0.1764). The Gleason index (G) and Shannon index of leaf, shoot, and twigs showed the maximum species diversity. The endophytic infection rate was found to be 100% in leaves and shoot whereas 98% infection rate was found in twig. The endophytic fungal extract when evaluated for its antibacterial activities exhibited the inhibition range of 15-22 mm. The minimum inhibitory concentration showed the antibacterial potency at lower concentration of 12.5-25 µg/mL with the all gram positive and gram negative bacterial strains presently studied (**Table 2**). The antioxidant and free radical scavenging activity recorded depicted 99% inhibition at the concentration of 6.25-20 mg/mL. The total phenolic content was found to be 3.142-4.445±2.85 µg/mL compared with Standard Tannic Acid. The endophytic fungal extract was evaluated for antioxidant and free radical scavenging ability through DPPH, FRAP, FTC, TBA and TBA assay. There are reports of occurrence of bioactive compounds, including antifungal agents in *Xylaria* sp., from different hosts (Pongcharoen *et al.*, 2008).

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