

Distribution and diversity of endophytic fungi associated with three medicinal tree species from Eturnagaram Wildlife Sanctuary, TS, India

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ABSTRACT

Distribution and diversity of endophytic fungal species associated with three medicinal tree species *Phyllanthus emblica* Linn, *Terminalia chebula* Retz, and *Terminalia bellirica* Roxb, whose fruits constitute the *triphala* an Ayurvedic formulation was assessed, in terms of isolation rate (IR), colonization frequency (CF), absolute frequency (f), relative frequency (RF), and species diversity. Leaf, stem, and bark were selected for the isolation of endophytic fungi. A large number of fungal species representing *Hyphomycetes*, *coelomycetes*, and sterile mycelia were found to be associated with different plant parts. *Hyphomycetes* dominated in all the fungi. Maximum IR was recorded in bark. Diversity indices (Shannon–Wiener and Simpson) varied with the tree species. The diversity richness of fungal endophytes points out facts that these fungal endophytes may play a significant role in plant health, metabolism, and medicinal attributes.

1. INTRODUCTION

Endophytic fungi colonize internal plant tissues without causing observable harm to their host. Fungal endophytes are a diverse group forming associations with almost all plants of the plant kingdom. They have been isolated from diverse plants including trees, fodders, vegetables, fruits, and medicinal plants [1]. Their biological diversity is enormous, especially in temperate and tropical vegetation's. Fungal endophytes confer many benefits to host plants and enable them to withstand abiotic stresses such as drought [2] and metal contamination [3]. Endophytes may also benefit host plants by preventing the colonization of pathogenic and predatory organisms. They have been found to be repertoire of novel organic compounds with broad spectrum of biological activities. Their secondary metabolites are particularly active because of their interaction with host [4]. The metabolites include varied structural groups such as terpenoids, steroids, xanthenes, chinones, phenols, isocoumarins, benzopyranones, tetralones, and enniatines [5] that are known to exhibit antibacterial, antiviral, antifungal, and anticancer activities [6].

Triphala is a popular, traditional Ayurvedic formulation consisting of equal parts of three myrobalans, namely, Amla (*Phyllanthus emblica*

Linn.), Harada (*Terminalia chebula* Retz.), and Bahera (*Terminalia bellirica* Roxb.). *Charaka Samhita*, an authoritative treatise on Ayurvedic medicine, has mentioned the potential uses of *Triphala* to treat any kind of disease. In combination, the three tree myrobalans exhibit anti-cleansing property including blood, reduce blood pressure, decrease levels of cholesterol, and removes harmful toxins from liver [7]. In view of wide range of well know medicinal properties of these three plants, it is proposed to investigate the distribution and diversity of endophytic fungal flora of these plants and their possible role in imparting the medicinal attributes to these three medicinal plants.

2. MATERIALS AND METHODS

2.1. Plant Materials

The selected plants for the present study is Amla (*Phyllanthus emblica* Linn; family: Phyllanthaceae), Harada (*Terminalia chebula* Retz; family: Combretaceae), and Bahera (*Terminalia bellirica* Roxb; family: Combretaceae), whose fruits together constitute the *Triphala*.

2.2. Collection of Plant Materials

The different plant parts, namely, stem, bark, and leaves of *P. emblica*, *T. chebula*, and *T. bellirica* (Each plant 60 samples) were collected from different locations of Eturnagaram wildlife sanctuary, Mulugu district, Telangana state, India. The Eturnagaram Wildlife Sanctuary is basically a tropical dry deciduous forest. It is regarded as one of

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the rich species diversity terrestrial ecosystems. The entire area of the Eturnagaram sanctuary is about 812 sq. km and the geographical coordinates, that is, latitude 18.3378° N and longitude 80.4299°E with an average temperature 28.3°C and rainfall of 821 mm/32.3 in/year. From this thick, forest healthy and disease free plants were selected for sampling. The plant parts were carefully collected and brought to the laboratory in sterilized polythene bags and processed within 24 h.

2.3. Isolation and Morphological Identification of Endophytic Fungi

The collected samples were first cleaned thoroughly in gentle running tap water to remove dirt and debris [8]. The stem, bark, and leaves were cut into segments (0.5–1 cm) and surface sterilized by modified method of Dobranic *et al.* (1995) [9]. The samples were immersed in 75% ethanol for 60 s, followed by 4% sodium hypochlorite for 180 s and then again in 75% ethanol for 30 s. Later, the segments were washed with sterile distilled water for 3 times and the excess moisture was blotted on Whatman no.1 filter paper. The surface sterilized segments were seeded in Petri dishes containing potato dextrose agar (PDA) medium supplemented with streptomycin.

The seeded Petri dishes were incubated at $26 \pm 1^\circ\text{C}$ and maintained dark-light cycles for 5–7 days. Sterilization and inoculations were performed in laminar air flow.

The pure colonies of fungi growing out of the segments were sub cultured on separate PDA slants and stored at 4°C for further process. The fungi were identified based on the morphology of cultural characteristics and direct microscopic observations of hyphae, reproductive structures and with the help of standard manuals and monographs [10,11].

2.4. Data Analysis

2.4.1. Colonization frequency (CF)

The CF of endophytic fungi isolated from the three tree species was calculated by following formula suggested by Suryanarayana *et al.* (2003) [12] and expressed in percentage.

$$\text{CF}\% = \frac{\text{Number of segments colonized by the fungi}}{\text{Total number of segments observed}} \times 100$$

2.4.2. Relative frequency (RF)

RF used to represent fungal density was calculated as described by Huang *et al.* (2008) [13].

$$\text{RF}\% = \frac{\text{Number of isolates of a species}}{\text{Total number of isolates}} \times 100$$

2.4.3. Isolation rate (IR)

IR, the measure of fungal richness of a sample was calculated as the number of isolates obtained from tissue segments divided by the total number of segments expressed, as fractions [14].

$$\text{IR} = \frac{\text{Number of isolates obtained from tissue segments}}{\text{Total number of segments}}$$

2.4.4. Species diversity

Simpson's Index of diversity was calculated using the following formula.

$$D = \frac{1 - \sum n(n-1)}{N(N-1)}$$

Where,

n = The total number of organisms of a particular species

N = The total number of organisms of all species

Shannon–Wiener diversity index was calculated using the following formula.

S

$$H_s = \sum (P_i) (\ln P_i)$$

Where, $i = 1$

H_s : Symbol for the diversity in a sample of S species or kinds

S: The number of species in the sample

P_i : Relative abundance of i^{th} species or kinds measures, $= n/N$

N: Total number of individuals of all kinds

n_i : Number of individuals of i^{th} species

In: Log to base 2

3. RESULTS AND DISCUSSION

The results obtained in the present investigations are presented in Tables 1 and 2, and Figures 3-6. A total of 348 fungal isolates were obtained from

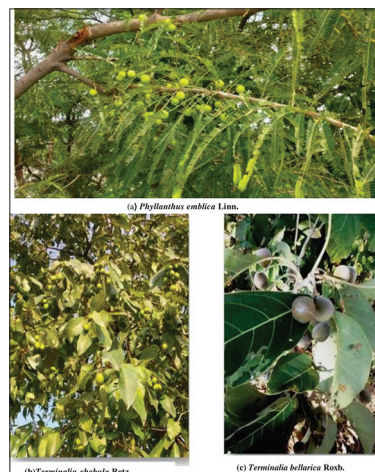


Figure 1: Morphology of Triphalia tree species.

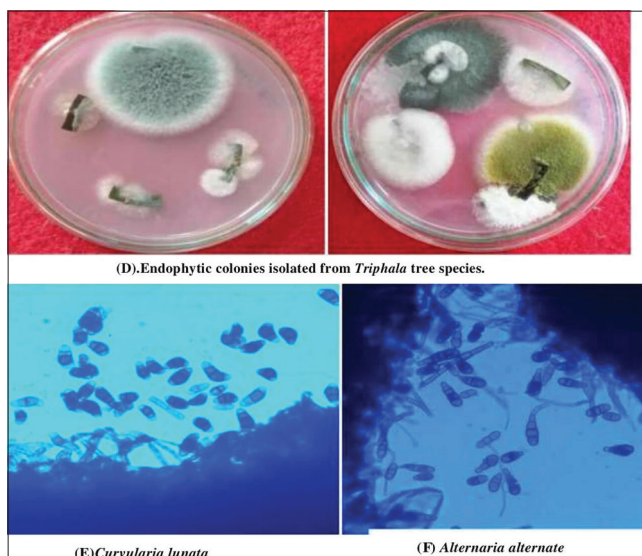


Figure 2: Colony morphology and microphotographs of endophytic fungi.

Table 1: Colonization frequency of endophytic fungal species isolated from *Triphala* tree species.

Endophytic fungi	<i>Phyllanthus emblica</i>						<i>Terminalia chebula</i>						<i>Terminalia bellirica</i>											
	Leaf		Stem		Bark		Leaf		Stem		Bark		Leaf		Stem		Bark		Leaf		Stem		Bark	
	NOI	CF%	NOI	CF%	NOI	CF%	NOI	CF%	NOI	CF%	NOI	CF%	NOI	CF%	NOI	CF%	NOI	CF%	NOI	CF%	NOI	CF%	NOI	CF%
<i>Acremonium strictum</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Alternaria alternata</i>	-	-	-	-	-	-	5	8.3%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. fasciculata</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Aspergillus flavus</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. fumigatus</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. niger</i>	3	5%	-	-	4	6.6%	4	6.6%	6	10%	3	5%	-	-	6	10%	2	3.3%	1	1.6%	2	3.3%	1	1.6%
<i>A. nidulans</i>	-	-	-	-	1	1.6%	-	-	-	-	1	1.6%	-	-	-	-	-	-	-	-	-	-	-	-
<i>A. terreus</i>	-	-	-	-	2	3.3%	2	3.3%	2	3.3%	3	5%	-	-	-	-	-	-	-	-	-	-	-	-
<i>Chaetomium globosum</i>	1	1.6%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cladosporium cladosporioides</i>	5	8.3%	2	3.3%	2	3.3%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. herbarum</i>	6	10	-	-	-	-	-	-	-	2	3.3%	2	3.3%	-	-	-	-	-	-	-	-	-	-	-
<i>Colletotrichum falcatum</i>	4	6.6%	4	6.6%	-	-	-	-	-	1	1.6%	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Curvularia lunata</i>	-	-	-	-	-	-	-	-	-	-	-	2	3.3%	-	-	-	-	-	-	-	-	-	1	1.6%
<i>C. spicifera</i>	1	1.6%	-	-	-	-	2	3.3%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. inaequalis</i>	-	-	-	-	2	3.3%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cylindrophora apiculata</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Drechslera spicifera</i>	-	-	-	-	2	3.3%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Fusarium dimerium</i>	-	-	-	-	3	5%	-	-	8	13.3%	-	-	12	20%	-	-	-	-	-	-	-	-	-	-
<i>F. semitectum</i>	2	3.3%	2	3.3%	2	3.3%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>F. sporotrichioides</i>	4	6.6%	3	5%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>F. solani</i>	-	-	-	-	-	-	3	5%	-	-	2	3.3%	-	-	-	-	-	-	-	-	-	-	-	-
<i>Nigrospora sphaerica</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Penicillium chrysogenum</i>	2	3.3%	-	-	3	5%	-	-	-	-	1	1.6%	4	6.6%	4	6.6%	7	11.6%	-	-	-	-	-	-
<i>P. citrinum</i>	3	5.6%	1	2.5%	1	2.5%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. expansum</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. notatum</i>	-	-	-	-	-	-	-	-	-	-	2	3.3%	2	3.3%	-	-	-	-	-	-	-	-	-	-
<i>P. rubrum</i>	-	-	-	-	4	6.6%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Trichoderma harzianum</i>	1	1.6%	2	3.3%	6	10%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>T. viride</i>	4	6.6%	-	-	4	6.6%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Phoma destructiva</i>	-	-	-	-	-	-	-	-	-	-	2	3.3%	1	1.6%	-	-	-	-	-	-	-	-	-	-
<i>P. glomerata</i>	4	6.6%	-	-	4	6.6%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Phyllosticta vaccinii</i>	-	-	3	5%	-	-	-	-	10	16.6%	-	-	3	5%	4	6.6%	2	3.3%	-	-	-	-	-	-
Sterile mycelium I	5	8.3%	2	3.3%	2	3.3%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(Contd..)

Table 1: (Continued)

Endophytic fungi	Phyllanthus emblica						Terminalia chebula						Terminalia bellirica							
	Leaf		Stem		Bark		Leaf		Stem		Bark		Leaf		Stem		Bark			
	NOI	CF%	NOI	CF%	NOI	CF%	NOI	CF%	NOI	CF%	NOI	CF%	NOI	CF%	NOI	CF%	NOI	CF%		
Sterile mycelium II	4	6.6%	1	1.6%	1	1.6%	6	10	-	-	-	-	-	-	-	-	-	-	-	
Sterile mycelium III	-	-	-	-	-	-	-	-	-	-	3	5%	-	-	-	-	-	-	-	
Sterile mycelium IV	-	-	3	5%	3	5%	3	5%	-	-	2	3.3%	-	-	-	-	-	-	-	
Sterile mycelium V	4	6.6%	-	-	3	5%	-	-	-	-	-	-	5	8.3%	3	5%	4	6.6%	19	5.4
Sterile mycelium VI	-	-	-	-	-	-	-	-	-	-	-	-	7	11.6%	-	-	9	15%	16	4.5
Total	47	88.2%	20	33.9%	52	90%	50	74.8%	21	24.7%	35	58%	49	79.4%	30	56.2%	44	59.4%	348	

540 segments were plated for frequency analysis; NOI: number of isolates; CF%: colonization frequency; f: absolute frequency; RF%: relative frequency.

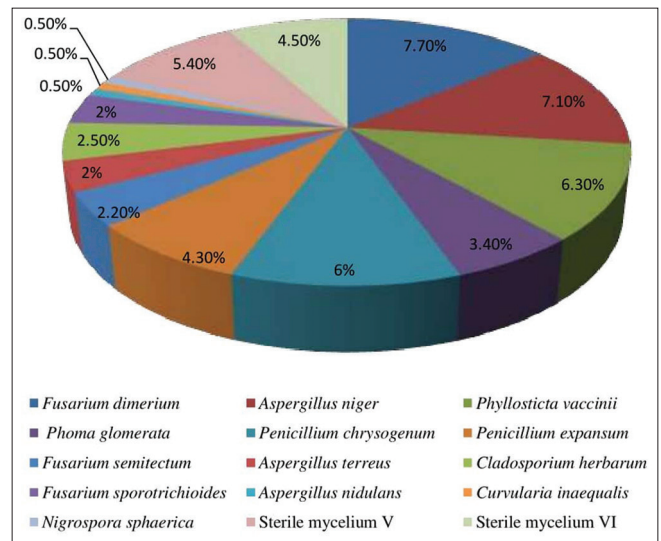


Figure 3: Relative frequencies of different endophytic fungal taxa isolated from Triphala trees.

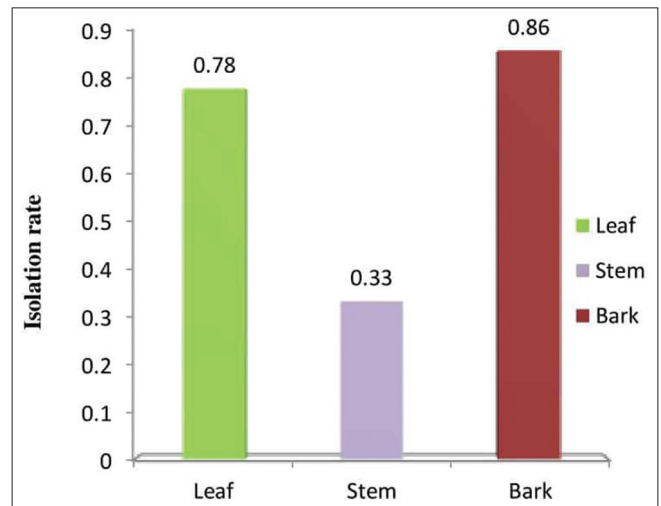


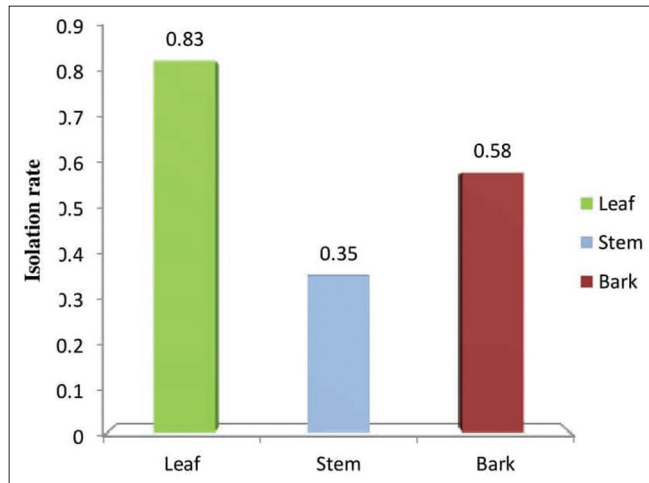
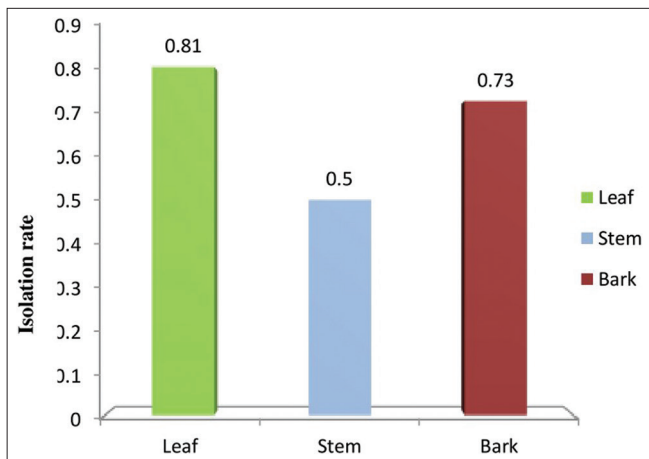
Figure 4: Isolation rate of fungal endophytes from P. emblica.

540 segments of three tree species parts, namely, leaf, stem, bark of *P. emblica*, *T. chebula*, and *T. bellirica* [Table 1]. They represented to 38 species which were belonging to 16 genera, in which 27 hyphomycetes, four coelomycetes, one ascomycetes, and six non-sporulating sterile mycelia. In *P. emblica*, 25 species belongs to 12 genera whereas, in leaves highest CF was shown by *Cladosporium herbarum* (10%) and least was shown by *Chaetomium globosum* and *Curvularia spicifera*, *Trichoderma harzianum* (1.6%). CF of *Colletotrichum falcatum* (6.6%) was highest in stem and least was in sterile mycelium II (1.6%). In bark, highest CF was recorded by *Trichoderma harzianum* (10%) and least was in *Aspergillus nidulans* and sterile mycelium (1.6%). In *T. chebula*, 18 species belonged to 12 genera. In the leaves, highest CF was recorded for *Phyllosticta vaccinii* (16.6%) and least for *Aspergillus terreus* and *Curvularia spicifera* (3.3%) and CF of *Aspergillus niger* (5%) was highest in stem and least was shown by *Penicillium chrysogenum* and *Colletotrichum falcatum* (1.6%), whereas in bark, the highest CF was recorded for *Fusarium dimerium* (20%) and least was in *Aspergillus nidulans* and *Phoma destructiva* (1.6%). In *T. bellirica* 22 species belonged to 12 genera. In the leaves, the highest CF was recorded for sterile mycelium VI (11.6%) and least for *Fusarium semitectum* (1.6%)

Table 2: Diversity indices of endophytic fungi isolated from *Triphala* trees species.

Indices	<i>Phyllanthus emblica</i>			<i>Terminalia chebula</i>			<i>Terminalia bellirica</i>		
	L	S	B	L	S	B	L	S	B
Simpson's dominance	0.32	0.15	0.36	0.17	0.12	0.10	0.19	0.05	0.20
Simpson's diversity	0.92	0.82	0.98	0.83	0.78	0.82	0.86	0.77	0.88
Species richness	16	9	19	10	8	9	11	10	12
Shannon-Wiener	2.75	1.96	2.81	2.10	1.88	1.90	2.05	1.68	2.25

L: Leaf; S: Stem; B: Bark

**Figure 5:** Isolation rate of fungal endophytes from *T. chebula*.**Figure 6:** Isolation rate of fungal endophytes from *T. bellirica*.

and CF of *Penicillium chrysogenum* (11.6%) was highest in stem and least for *Curvularia lunata* and *Alternaria fasciculata* (1.6%). In bark, the highest CF was recorded by *Penicillium expansum* (20%), followed by sterile mycelium VI (15%) and least was shown by *Acremonium strictum*, *Aspergillus niger*, *Curvularia lunata*, *Cylindrophora apiculata*, *Drechslera spicifera*, *Fusarium semitectum*, and *Penicillium rubrum* (1.6%), respectively [Table 1].

Among three tree species of *Triphala*, the RF of endophytic fungal genera is highest in *Fusarium dimerium* (%RF = 7.7%), followed by *Aspergillus niger* (%RF = 7.1%) and the least percentage of frequency present in *Aspergillus nidulans*, *Curvularia inaequalis*, and *Nigrospora sphaerica* (%RF = 0.5%) [Figure 3].

In the plant parts, namely, leaves, stem, and bark of *P. emblica* maximum fungal endophytic IR were recorded in bark (0.86) and the lowest IR was recorded in stem (0.33). However, in *T. chebula*, the highest IR was observed in leaves (0.83), and lowest IR was recorded in stem (0.35). In *T. bellirica*, leaves (0.81) were occupied by highest IR and stem (0.5) was recorded with least IR [Figures 4-6].

Shannon–Wiener and Simpson diversity indices of fungal endophytes varied within three tree species. In *P. emblica*, high Shannon–Wiener diversity index was recorded in bark ($H^1 = 2.81$), low indices were recorded in stem ($H^1 = 1.96$), where Simpsons index was high in bark with a richness of 19 fungal species. For *T. chebula* high Shannon–Wiener diversity index was recorded in leaves ($H^1 = 2.10$), low indices were recorded in stem ($H^1 = 1.88$), and Simpsons index was high in leaves with a richness of ten fungal species. In *T. bellirica*, high Shannon–Wiener diversity index was recorded in bark ($H^1 = 2.25$), low indices were recorded in stem ($H^1 = 1.68$), and Simpsons index was high in bark with a richness of 121 fungal species [Table 2].

Triphala, an Ayurvedic herbal rasayana churna, is known as one of the oldest forms of health care, basically originated in India and it is called as a complete body cleanser. It also purifies blood, reduces blood pressure, and decreases level of cholesterol. Recent studies reveal that many tree species especially medicinal in nature are associated with many fungal endophytes. Endophytic fungi are one of the most unexplored and diverse group of organisms having symbiotic association with higher life forms and may produce beneficial substances to host [15]. The plant tissues, especially leaves and stems, are excellent reservoirs for endophytic fungi [16]. In this context, the present investigations aimed to isolate the endophytic fungi from *Triphala* tree species (*P. emblica*, *T. chebula*, and *T. bellirica*), to assess their possible role in imparting medicinal properties.

Three tree species were selected for the study from different location of Eturnagaram forest area, stretching over 25 km distance from sampling to sampling. The results of the present study reveal that three tree species of triphala plants are associated with different endophytic fungi belonging to diverse taxonomic groups. From 540 segments of plant material a total 348 isolates were obtained were belongs to 38 fungal taxa, several researchers working on different plants have also noted the association of endophytic fungi [1]. However, a wide variation has been observed in there CF between three plant segments, namely, leaves, stem, and bark. Where, *Aspergillus*, *Fusarium*, and *Penicillium*, *Phyllosticta* species, and sterile mycelium dominated. The IR also varied in these three plant parts. Diversity indices for fungal endophytes analyzed by Simpson and Shannon–Wiener indices indicated differences in endophytic fungal isolates and species richness [17]. Some of the endophytic fungi reported through this study may be explored for the secondary metabolites and used in pharma and agri-industries. The endophytic fungi isolated from three tree species of *triphala* from Eturnagaram forest area are rich with fungal diversity.

4. CONCLUSION

The present investigations reveal that the three medicinal plants are associated with large and diverse fungal endophytes in its different parts. The fungal species belonged to different taxonomic groups especially of hyphomycetes. The endophytic fungal distribution varied with the tree species. The contribution of these endophytes in the metabolism and health of the plant needs to be investigated in detail.

5. ACKNOWLEDGMENT

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6. CONFLICTS OF INTEREST

All the authors declare that there are no conflicts of interests regarding the publication of this paper.

7. FINANCIAL SUPPORT AND SPONSORSHIP

None.

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