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Evaluation of antioxidant and antibacterial activities of endophytic fungi isolated from *Bauhinia racemosa Lam* and *Phyllanthus amarus* Schum and Thonn.

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ABSTRACT

The objective of this study is to screen the antioxidant and antibacterial activity of endophytic fungi isolated from surface sterilized leaves, stem and root of Bauhinia racemosa lam and phyllanthus amarus Shum and Thonn. 13 fungal species of endophytic fungi were isolated including Colletotrichum gleosporioides, Drechslera halodes, Nigrospora sphaerica, Phoma glomerata, from Bauhinia racemosa and Aspergillus nidulans, Chaetomiun spiralis, Colletotrichum circinans, Colletotrichum crassipes, Colletotrichum falcatum, Collectotrichum truncatum, Penicillium citrinum, Phoma chrysanthemicoli and Phyllosticta Sp from Phyllanthus amarus. The dominant fungi were evaluated for its antioxidant and antibacterial activity. Both the entophyte and the host extracts were extracted by water and methanol and tested for their total phenolic content and radical scavenging potentials. The fungal extracts were also assessed for antibacterial activity against bacterial strains: Bacillus subtilis and Escherichia coli. The results of the MIC study showed that the Chaetomium spiralis extracts exhibited higher activity against bacteria, Escherichia coli.

Key words: Antioxidant, Antibacterial activity, Total Phenolic Content, Radical scavenging potentials.

INTRODUCTION

Endophytic fungi are the microorganisms that are present in the living tissues of various plants, establishing a mutual relationship without causing any symptom of diseases. Endophytes are rich sources of bioactive metabolites, which have important potentials in medicine, agriculture and industries [1]. Endophytes are known to produce metabolites such as alkaloids, terpenoids, steroids, quinones, isocoumarin derivatives, flavanoids, phenols, phenolic acids, and peptides. Some species produce novel antimicrobial agents and other produce potent anti-cancer compounds (Taxol from *Taxomyces andreanae*) and yet others produce compounds that can be utilized industrially, such as enzymes and solvents [2]. Since, the plant tissue where the endophytes exist is a eukaryotic system, it would appear that the secondary metabolites produced by the endophytes may have reduced cell toxicity; otherwise, death of the host tissue may occur. Thus, the host itself has naturally served as a selection system for microbes having bioactive molecules with reduced toxicity toward higher organisms [3].

The plant *Bauhinia racemosa* (L) belongs to the *Caesalpiniaceae* family. It occurs frequently in India, Ceylon, China, and Timor. The stem bark of the plant is an astringent and is used in the treatment of headache, fever, skin diseases, tumours, blood diseases, dysentery, and diarrhoea [4]. The fresh flower buds of the plant showed anti-ulcer activity [5, 6]. Flowers, buds and dried leaves are used to treat dysentery. Root bark is used in inflammation of liver [7, 8]. Seeds are tonic and aphrodisiac. Leaves have antidiabetic Action [9 & 10]. The seeds contain crude protein 16.8; crude lipid 4.9; crude fibre 6.5; total carbohydrates 67.9 g/100g. The mineral composition of the seed is:

sodium 24.5; potassium 1013.8 ; calcium 708.8 ; magnesium 264.8 ; phosphorus 326.7 ; iron 19.9 ; copper 0.3 ; zinc 8.9 ; and manganese 2.2 mg/100g. The albumins of the seed exhibited no haem agglutination activity, whereas globulins showed weak haem agglutination, trypsin inhibitor activity. The leaves are having antimalarial activity [11].

Phyllanthus amarus is a plant of the family Euphorbiaceae and has about approximately 800 species which are found in tropical and subtropical countries of the world. The name '*Phyllanthus*' means "leaf and flower" and named so because of its appearance where flower, fruit and leaf appears fused. *Phyllanthus amarus* is a branching annual glabrous herb which is 30-60 cm high and have slender, leaf-bearing branch lets, distichous leaves which are sub sessile elliptic-oblong, obtuse, rounded base. Flowers are yellowish, whitish or greenish, auxiliary, male flowers in groups of 1-3 whereas females are solitary. Fruits are depressed-globose like smooth capsules present underneath the branches and seeds are trigonous, pale brown with longitudinal parallel ribs on the back. The plant has been found in Philippine, Cuba, Nigeria and among others. In India, *Phyllanthus amarus* is widely distributed as a weed in cultivated and waste lands [12].

EXPERIMENTAL SECTION

Collection of Plant material

Bauhinia racemosa Lam and *Phyllanthus amarus* Schum and Thonn were collected from the Garden of Bannari Amman Institute of Technology, Sathyamangalam, Tamil Nadu (India). The disease free parts of the plant are collected and transferred to the laboratory in a sterile polythene bag and it was processed within 24 hours.

Isolation of endophytic fungi

Stems and roots of each sample were rinsed with water and surface-disinfected by immersion in 75% ethanol for 1 min, 5 min in 5% sodium hypochlorite solution, and 1 min in sterile de-ionized water for three times. The samples were then surface-dried with sterile filter paper [13]. Roots and stems were cut into 0.5 cm x 0.5 cm pieces and placed in petri dishes with potato dextrose agar (PDA) medium (g/l; dextrose-20, agar-15, potato infusion-200) and cultured at 25^{o}C under dark [14].

The purified endophytic isolates were transferred separately to PDA slants and accessioned accordingly depending upon the plant and plant parts from which they have been isolated. Finally, all the purified entophytes were maintained at 4^{o}C till further used.

Identification of endophytic fungi

Identification of fungal endophytes was carried out based on the morphology of surface texture and spores at the hyphal tips with standard manual [15]. The fungal isolates on sterile slides were stained with Lacto phenol Cotton Blue and visualized in research microscope. Some endophytic fungi do not produce spores and it was grouped under a species named "Sterile form" [16].

Statistical Analysis

Colonization frequency

The percentage of colonization frequency (CF) was calculated as [16] as follows:

$$CF\ (\%) = \frac{\text{Number of species isolated}}{\text{Number segments screened}} \times 100$$

Relative Percentage Occurrence (RPO) of Different groups of Fungi

Relative Percentage Occurrence (RPO) of each group (viz., Ascomycetes, Hyphomycetes, Coelomycetes and Sterile forms) of fungal species in each plant species was calculated as follows:

$$RPO = \frac{\text{Density of colonization of one group}}{\text{Total Density of colonization}} \times 100$$

Endophytic Infection Rate

$$EIR\ (\%) = \frac{\text{Number of infected segments}}{\text{Total number of segments screened}} \times 100$$

Fungal Cultivation

The endophytic fungi were cultured in 500 ml flasks, each containing 150 ml potato dextrose liquid medium (g/l; dextrose-20, potato infusion-200). Each fungus were inoculated and cultured with shaking (120 rpm) at 25^0C under dark conditions for 1 week. After that, the cultures were filtered. The mycelia was filtered and transferred to a glass petri plate and dried overnight in a hot air oven at 40°C. The content of the dry mycelia was powdered using sterilized mortar and pestle. The contents were transferred to pre-weighed polyethylene zip-lock covers and stored at 4^oC. 0.1 g of dry powder was extracted in 10 ml of water and methanol separately and designated as aqueous and methanolic extracts respectively.

Antioxidant activity

Estimation of total phenolic content

Total phenolic content of fungal mycelia was determined by Folin-Ciocalteau (FC) method by taking Gallic acid as a standard of 1mg/ml [17]. Different concentrations of standard as well as the water and methanolic extracts were taken and one ml of FC reagent (1:1 dilution) was added. After 3- 5 min, 2.0 ml of sodium carbonate (20%, w/v) was added and the mixture was allowed to stand for 45 min under dark condition. After the incubation period, the absorbance was read at 765 nm using Spectrophotometer.

DPPH radical scavenging assay

Different aliquots of aqueous and methanolic extracts of plant sources and fungal extract were taken and the total volume was made up with water and methanol respectively. One 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 [18]. The per cent radical scavenging was calculated as follows:

% Radical scavenging = A_c-A_s (100)/ A_c

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

Antibacterial activity

Agar- Well Diffusion Method

The antimicrobials present in the fungal extract are allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The fungal strains used were *Chaetomium spiralis*, *Drechslera* Sp, *Nigrospora* Sp and *Phoma* Sp. Test microorganisms used were *Escherichia coli* and *Bacillus subtilis*. The resulting zones of inhibition will be uniformly circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeters.

Petri plates containing 20ml Nutrient agar medium were seeded with the 24hr culture of bacterial strains. Wells were cut and 20 µl of the fungal extracts were added. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well [19].

Minimum Inhibitory Concentration (MIC)

The minimum concentrations of the fungal extract to inhibit the microorganisms were also determined by a micro dilution method using bacterial fractions serially diluted in sterile nutrient broth [20]. The antibacterial efficacy of fungal extracts was studied against two bacterial strains, i.e. *Bacillus subtilis* (MTCC 121), *Escherichia coli* (MTCC 443) procured from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India

RESULTS AND DISCUSSION

Isolation of Endophytic fungi from *Bauhinia racemosa* and *Phyllanthus amarus*

Two fifty segments (approx.0.5cm^2) from each of the leaf, root and stem tissues of *Bauhinia racemosa* Lam and *Phyllanthus amarus* Schum and Thonn (Figure 1) were sterilized and screened for the presence of endophytic fungi (Figure 2).

Figure 1. Habitat of *Bauhinia racemosa*

Figure 4. Habitat of *Phyllanthus amarus*

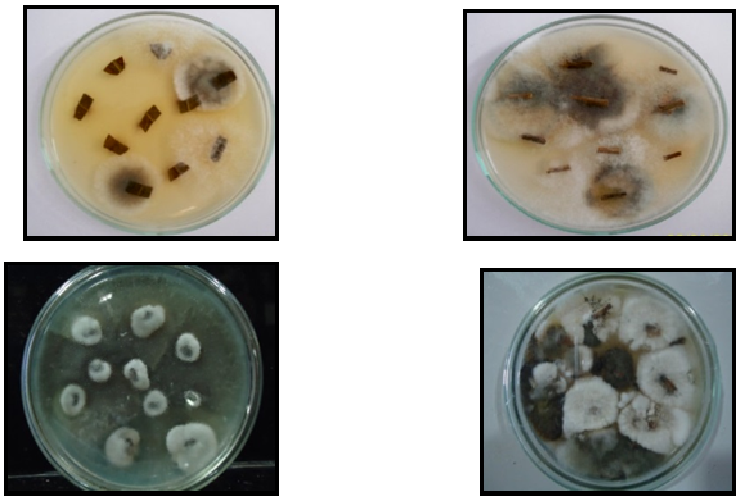
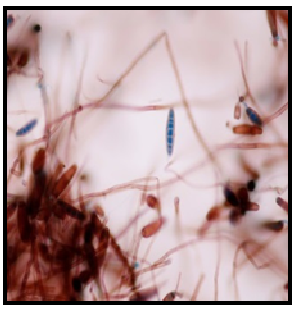


Figure 2. Endophytic fungal propagules emerging from tissues of selected medicinal plants

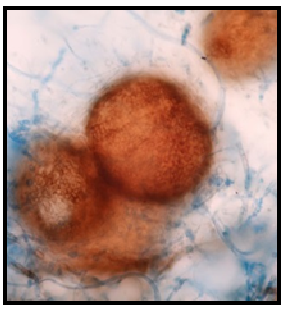
Identified endophytic fungi from the medicinal plants:

A total of 250 endophytic fungal isolates which belongs to 9 species were identified from two medicinal plants. The endophytic fungi from the medicinal plants such as *Bauhinia racemosa* Lam and *Phyllanthus amarus* Schum and Thonn which were grouped under Ascomycetes, Coelomycetes, Hyphomycetes, Xylariales, and Sterile form (Table 1). *Chaetomium* Sp. (52%) belongs to Ascomycetes found in *Bauhinia racemosa* Lam. *Colletotrichum circinans* (14%), *Colletotrichum crassipes* (4%), *Colletotrichum falcatum* (26%), *Colletotrichum spiralis* (2%), *Colletotrichum truncatum* (10%), *Phoma chrysanthemicoli* (58%), *Phoma epicoccinia* (18%) and *Phyllosticta* Sp (16%) belongs to Coelomycetes (Figure 3) The percentage of colonization frequency of *Nigrospora sphaerica* (14%), *Drechslera halodes* (24%), *Aspergillus nidulans* (4%) and *Penicillium citrinum* (14%) which belongs to Hyphomycetes group. Shekhawat *et al.,* [21] found that Hyphomycetes widely occurs in plants and protect against pathogens. *Pestalotiopsis* Sp (20%) belongs to Xylariales in *Phyllanthus amarus* Schum and Thonn. The overall relative percentage occurrence of Sterile forms were maximum when compared to the Ascomycetes, Coelomycetes, Hyphomycetes and Xylariales (Figure.4). Lacap *et al.,* [22] reported that sterile mycelia were prevails in most of the endophytic research studies. The overall percentage of endophytic infection rate was 86% in *Bauhinia racemosa* Lam and 82.66 in *Phyllanthus amarus* Schum and Thonn (Figure 4 - 10).

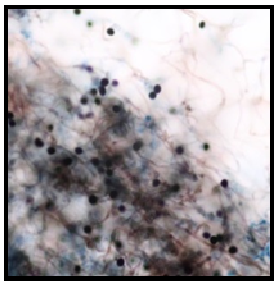
Chaetomium spiralis



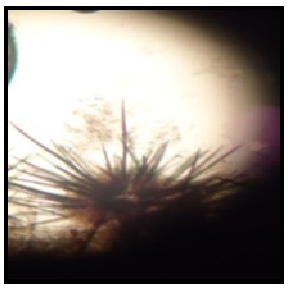
Drechslera Sp.



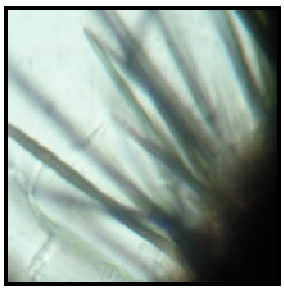
Nigrospora Sp.



Phoma Sp.



Colletotrichum crassipes



Colletotrichum circinans

Figure 3 .List of endophytic fungi identified from Bauhinia *racemosa* Lam and *Phyllanthus amarus* Schum and Thonn

Table.1: Colonization Frequency of endophytic fungi isolated from *Bauhinia racemosa* Lam and *Phyllanthus amarus* Schum and Thon.

S. No	Species	Colonization frequency in Percentage (%)				
		Bauhinia racemosa		*Phyllanthus amarus*		
		Leaf	Stem	Leaf	Stem	Root
	ASCOMYCETES					
1	*Chaetomium* sp.1	10	-			
2	*Chaetomium* sp.2	6	10			
3	*Chaetomium* sp.3	20	6			
	COELOMYCETES					
4	*Colletotrichum circinans*	-	-	14	-	-
5	*Colletotrichum* crassipes	-	-	4	-	-
6	*Colletotrichum falcatum*	-	-	26	-	-
7	*Colletotrichum spiralis*	2	-	-	-	-
8	*Colletotrichum truncatum*	-	-	10	-	-
9	*Phoma epicoccinia*	-	-	-	18	-
10	*Phoma chrysanthemicoli*	-	2	20	36	-
11	*Phyllosticta* sp.	-	-	-	16	-
	HYPHOMYCETES					
12	*Aspergillus nidulans*	-	-	-	4	
13	*Drechslera halodes*	14	10	-	-	-
14	*Nigrospora sphaerica*	10	4	-	-	-
15	*Penicillium citrinum*	-	-	-	-	34
	XYLARIALES					
15	*Pestalotiopsis* sp.	-	-	-	-	14
	STERILE FORMS					
16	Sterile form 1	5	6		1	8
17	Sterile form 2	7	6		2	
	Total number of Species	**6**	**5**	**5**	**7**	**4**
	Total number of isolates	**74**	**44**	**74**	**74**	**48**

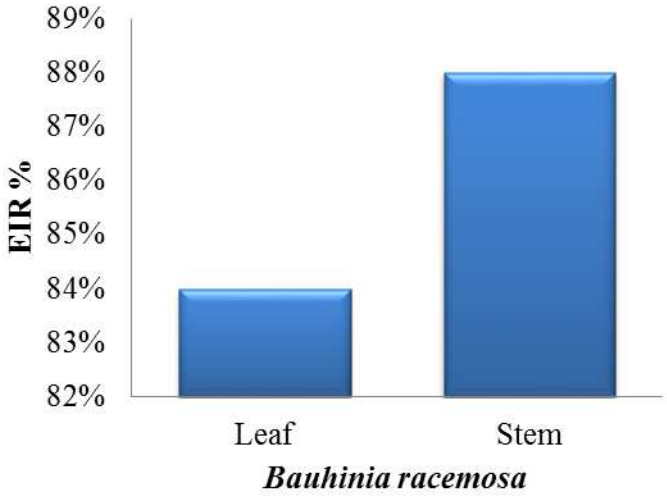


Figure 4. Endophytic infection rate of *Bauhinia racemosa*

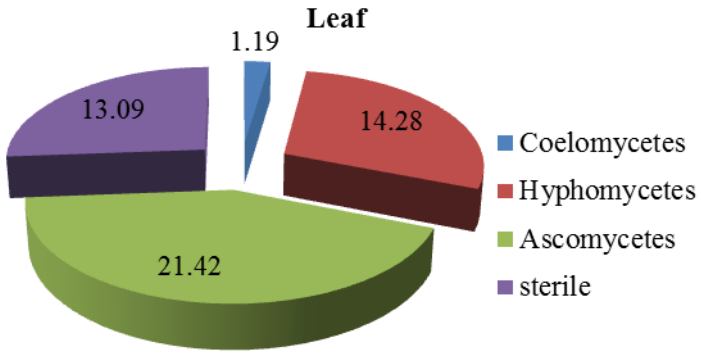


Figure 5. Relative Percentage Occurrence of different groups of endophytic fungi recorded from leaf tissues of *Bauhinia racemosa*

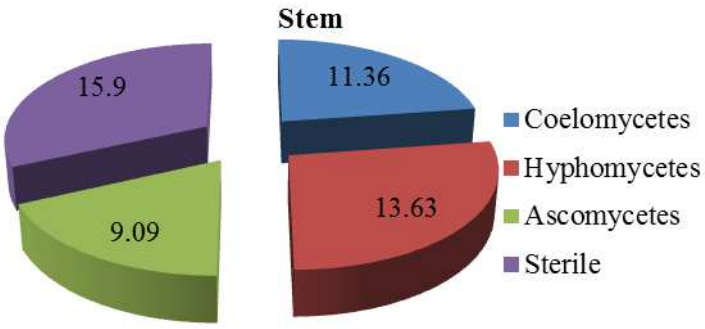


Figure 6. Relative Percentage Occurrence of different groups of endophytic fungi from stem tissues of *Bauhinia racemosa*

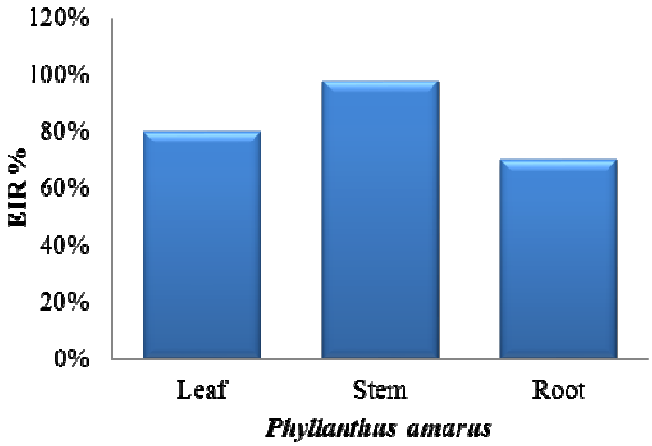


Figure 7. Endophytic infection rate of *Phyllanthus amarus*

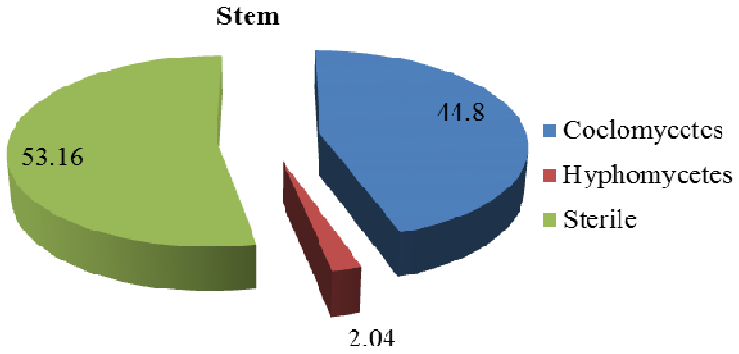


Figure 8. Relative Percentage Occurrence of different groups of endophytic fungi recorded from Stem tissues of *Phyllanthus amarus*

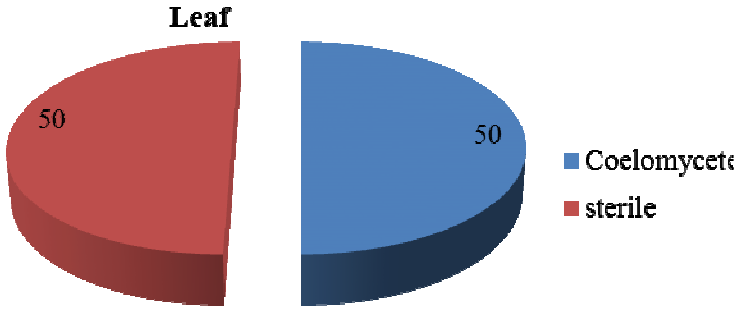


Figure 9. Relative Percentage Occurrence of different groups of endophytic fungi recorded from Leaf of *Phyllanthus amarus*

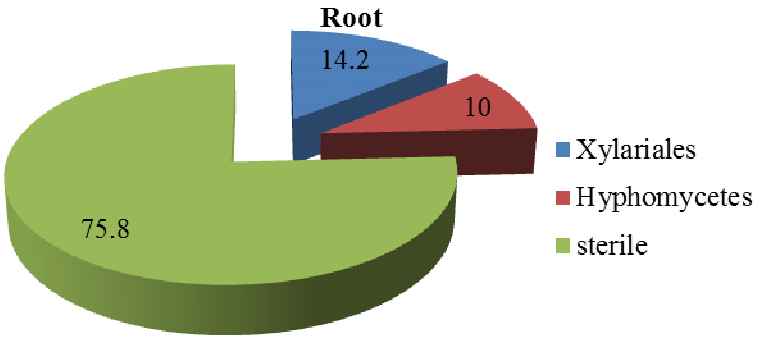


Figure 10. Relative Percentage Occurrence of different groups of endophytic fungi recorded from root tissues of *Phyllanthus amarus*

Determination of anti-oxidant activity in fungal and host extracts

The mycelial extract showed a lesser amount of phenolic as well as DPPH radical scavenging activity comparing to the host plant. The phenolic compounds are responsible for the antioxidant activity of host plant and its isolated endophytic fungus. The activity of phenolic compounds depends on their chemical structure. Methanolic extracts of *Drechslera* Sp. and *Nigrospora* Sp showed higher phenolic content and scavenging potentials, whereas *Phoma* Sp. and *Chaetomium spiralis* showed increased phenolic content in aqueous extract (Figure 11- 16). Host plant has higher activity when comparing to fungal extract.

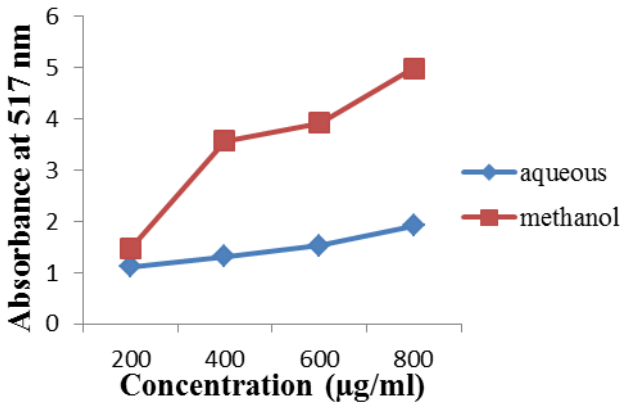


Figure 11. Total phenolic content of *Bauhinia racemosa* in aqueous and methanolic extract

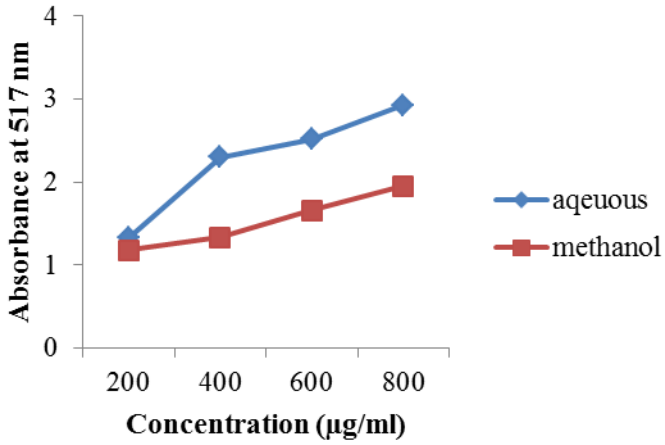


Figure 12. Total phenolic content of *Phyllanthus amarus* in aqueous and methanolic extract

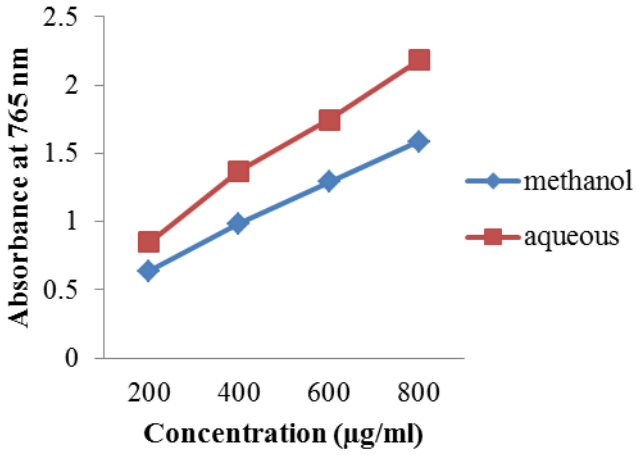


Figure 13. Total phenolic content in aqueous and methanolic extract of *Chaetomium spiralis*

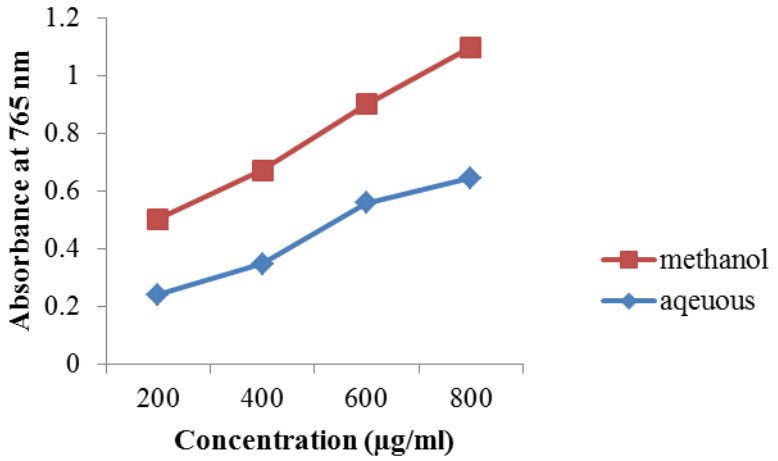


Figure 14. Total phenolic content of in *Drechslera* Sp. aqueous and methanolic extract

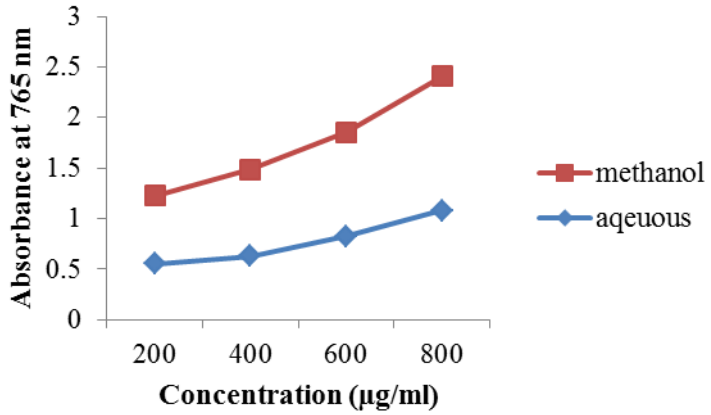


Figure 15. Total phenolic content of in *Nigrospora* Sp. aqueous and methanolic extract

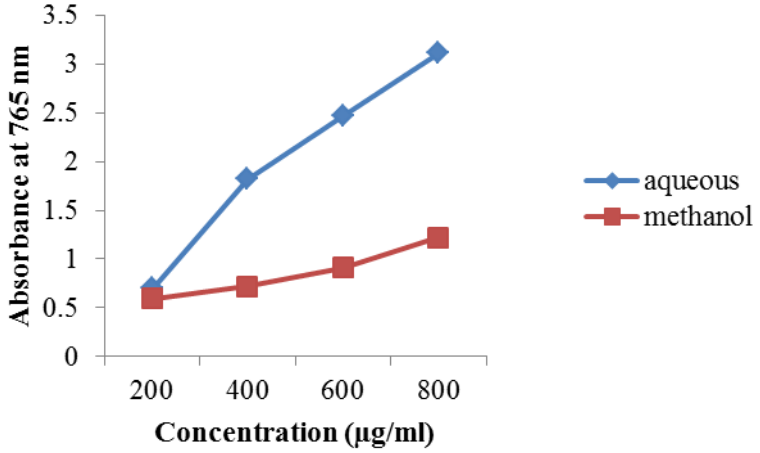


Figure. 16 Total phenolic content of in *Phoma* Sp. aqueous and methanolic extract

DPPH Radical scavenging activity

The radical scavenging activity of *Bauhinia racemosa* is 78.9% in aqueous extract and 72.5% in methanolic extract (Fig. 4.21). In *Phyllanthus amarus*, the scavenging potential is of 75% in aqueous and 77.8% in methanolic extract (Fig. 4.22). There is decrease in the scavenging potential of endophyte with respect to host. Maximum scavenging activity is found in *Drechslera* Sp. which is 67.73% in methanolic extract (Fig. 17 - 22).

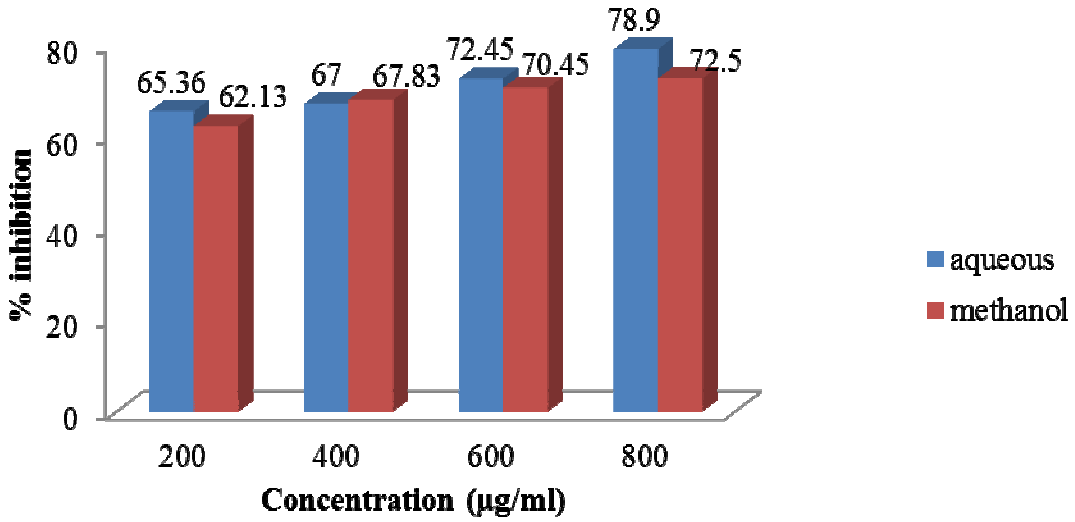


Figure 17. Percentage Inhibition of *Bauhinia racemosa*

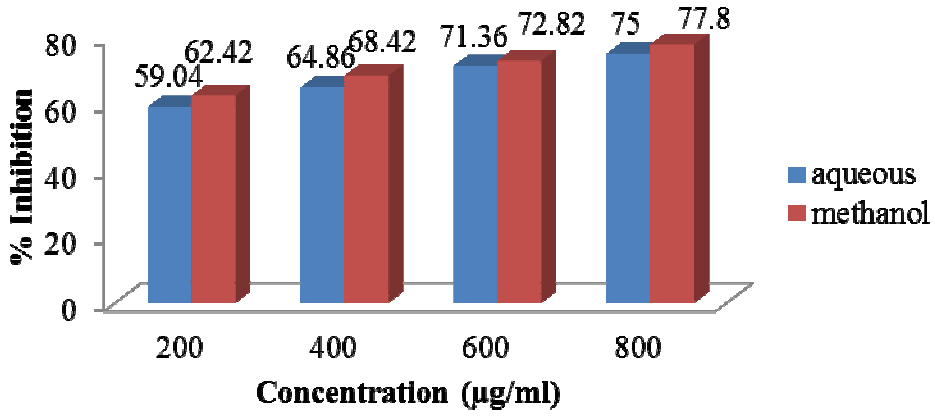


Figure 18. Percentage Inhibition of *Phyllanthus amarus*

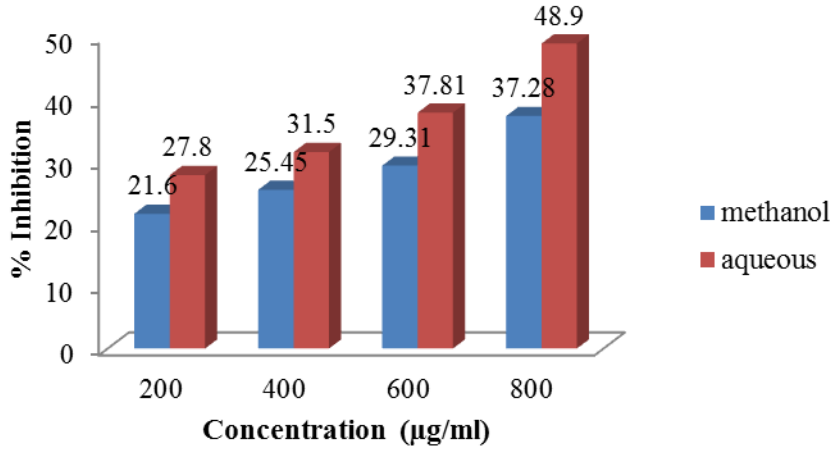


Figure 19. Percentage Inhibition of *Chaetomium spiralis*

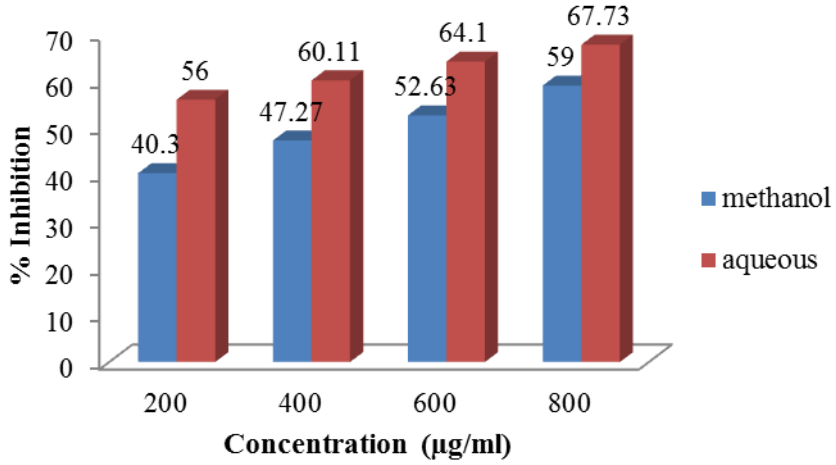


Figure 20. Percentage Inhibition of *Drechslera* Sp

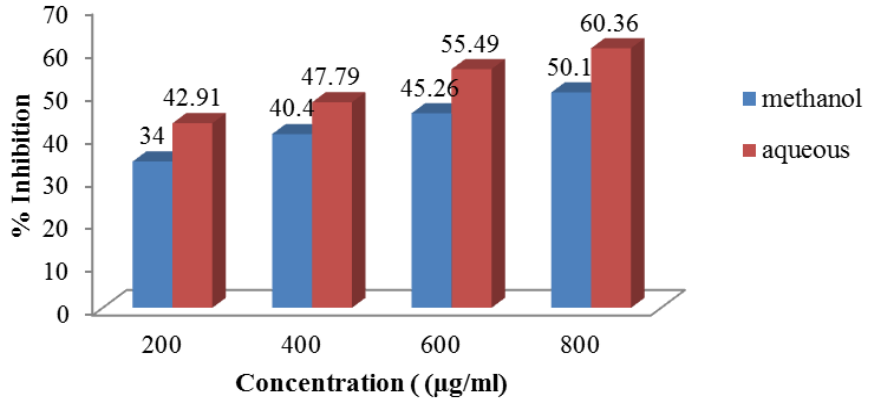


Figure 21. Percentage inhibition of *Nigrospora* Sp

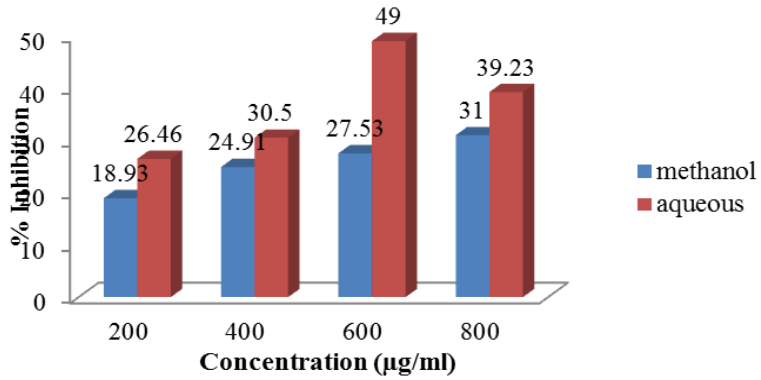


Figure 22. Percentage Inhibition of *Phoma* Sp

Antibacterial activity

Agar- Well Diffusion Method

The range of inhibition was found higher in *Chaetomium spiralis* (18 mm), against *Escherichia coli* (Fig. 27). The lowest concentration of antibacterial activity recorded was found in *Drecshlera* Sp. and *Nigrospora* Sp. against *Escherichia coli* (Fig. 29, Fig. 31). There is no inhibition recorded against *Bacillus subtilis* for *Drecshlera* Sp. and *Nigrospora* Sp (Figure 27 -34). No inhibition was found in *Phoma* Sp. (both *Escherichia coli* and *Bacillus subtilis*).

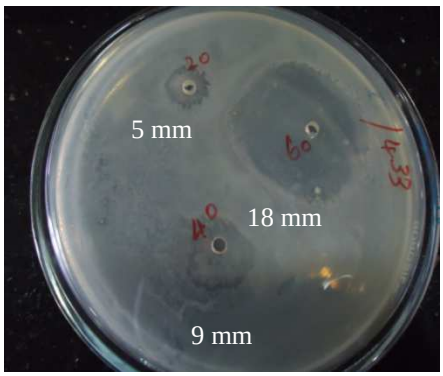


Figure 27. *Chaetomium spiralis*
***E.coli* (MTCC 443)**

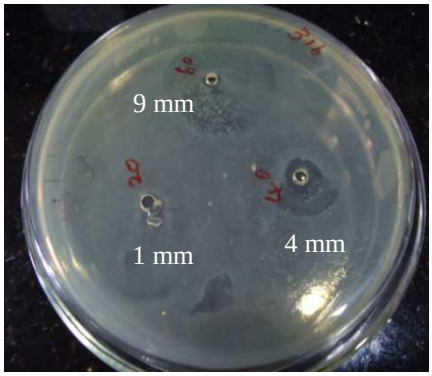


Figure 28. *Chaetomium spiralis*
***Bacillus subtilis* (MTCC 121)**

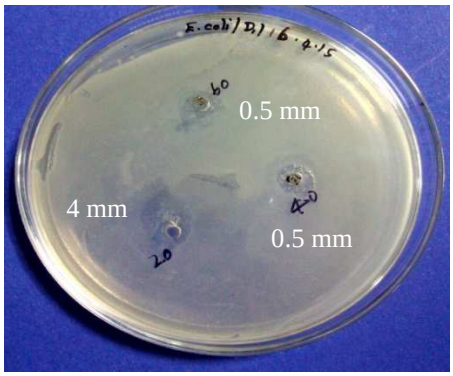


Figure 29. *Drechslera* Sp.
***E.coli* (MTCC 443)**

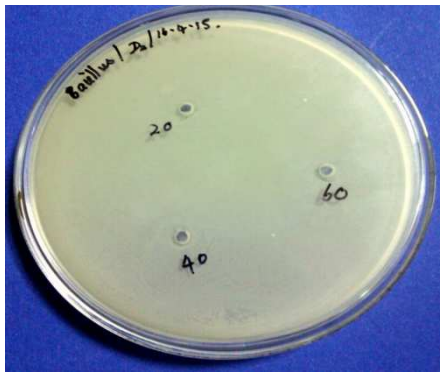


Figure 30. *Drechslera* Sp.
***Bacillus subtilis* (MTCC 121)**

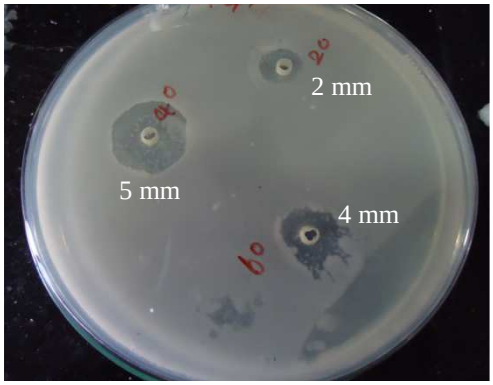


Figure 31. *Nigrospora* Sp
***E.coli* (MTCC 443)**

Figure 32. *Nigrospora* Sp
***Bacillus subtilis* (MTCC 121)**

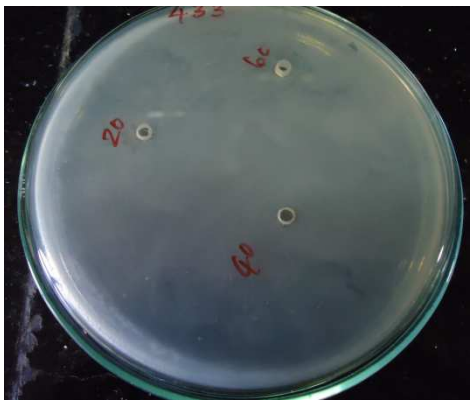


Figure 33. *Phoma* Sp
***E.coli* (MTCC 443)**

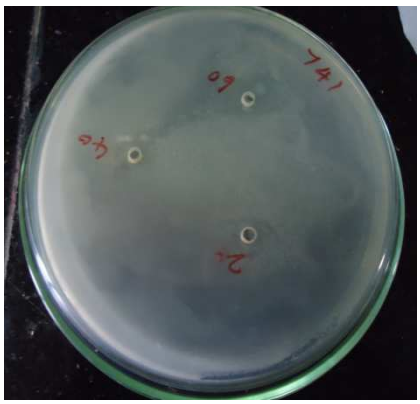


Figure 34. *Phoma* Sp
***Bacillus subtilis* (MTCC 121)**

Minimum Inhibitory Concentration (MIC)

The lowest concentration of MIC recorded was found in *Drecshlera* Sp. against *Escherichia coli*. No inhibition was found in *Phoma* Sp (both *Escherichia coli* and *Bacillus subtilis*). Similar results were observed with *Nigrospora* Sp. and *Drecshlera* Sp. against *Escherichia coli*. The maximum inhibition for both bacterial strains was found in *Chaetomium spiralis.* The details of MIC recorded for each species against the studied bacteria are presented in Table 2.

Table 2. Minimum Inhibitory Concentration (MIC)

S. No	Fungal Sample	Antibacterial activity (µg/ml)	
		Escherichia coli	*Bacillus subtilis*
1	*Chaetomium spiralis*	50%	25%
2	*Drechslera* Sp.	25%	Nil
3	*Nigrospora* Sp.	25%	Nil
4	*Phoma* Sp.	Nil	Nil

The study provides information on the diversity of endophytic fungi from two medicinal plants. It has also revealed that the aqueous and methanolic extracts of both plant and fungal mycelia showed good phenolic content and radical scavenging activity. The mycelial extract showed a lesser amount of phenolic as well as DPPH radical scavenging activity comparing to the host plant. The phenolic compounds are responsible for the antioxidant activity of host plant and its isolated endophytic fungus. The activity of phenolic compounds depends on their chemical structure. Better antioxidant activity was observed higher in aqueous extract of *Phyllanthus amarus* and methanolic extract of *Bauhinia racemosa*. A study of antioxidant activity with endophytic extracts indicated that the acetone extraction yielded good antioxidant activity methanol and water [23].Therefore, the biological extracts of entophytes would be used as alternatives to plant extracts. Out of 4 isolates, *Chaetomium spiralis* could inhibit few bacteria used in this study. Each of them displayed antimicrobial activity against at least one test microorganism with inhibition zones that ranged from 18 to 0.5 mm. The isolates inhibited strains of gram-negative bacteria better than gram-positive bacteria. The MIC values of crude methanolic extract from isolates are shown in Table 2. The result showed the fungal extract inhibited gram-negative than gram-positive bacteria.

CONCLUSION

The study provides information on the diversity of endophytic fungi from two medicinal plants. It has also revealed that the aqueous and methanolic extracts of both plant and fungal mycelia showed good phenolic content and radical scavenging activity. The mycelial extract showed a lesser amount of phenolic as well as radical scavenging activity comparing to the host plant. *Chaetomium spiralis* showed higher antibacterial activity whereas *Phoma* Sp. has no inhibition against bacteria. *Nigrospora* Sp. and *Drechslera* Sp. showed an average antibacterial activity. The study concludes the presence of bioactive compound in the extract which shows antioxidant and antibacterial activity in the screened fungal species.

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